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UNIVERSITY OF ALBERTA

Analysis of the expression patterns of the *fem-2* gene of *Caenorhabditis elegans*

BY

Troy Geoffery Johnson ©

A thesis submitted to the Faculty of Graduate studies and Research in partial fulfillment of the requirements for the degree of Master of Science

DEPARTMENT OF GENETICS

Edmonton, Alberta

Fall, 1995

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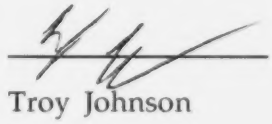

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Dr. H. McDermid

Dr. F. Nargang

Dr. S. Zalik

Date: June 16 1995.

To my Parents, Gary and Irene Johnson, who have always supported my decisions regarding school including the difficult decision to move to Alberta, even to the point of driving out with me.

ABSTRACT

fem-2 is an important gene in the control of male somatic sex determination and spermatogenesis in *Caenorhabditis elegans*. *fem-2* acts along with *fem-1* and *fem-3* to negatively regulate the terminal regulator of sex determination in the soma, *tra-1*. The aim of this study was to examine the temporal and spatial expression patterns of *fem-2*. Northern blot analysis revealed that low levels of *fem-2* are present in all developmental stages and significantly increased in the later four stages with no sex or stage specific differences observed. Expression patterns of a *fem-2/lacZ* reporter construct showed staining in the head, tail and intestine of both sexes and the distal tip cells of the male. *fem-1* and *fem-3* mutations altered the expression patterns in the distal tip cells. These results have helped to clarify the role of *fem-2* in sex determination and provided insight into when and where *fem-2* is required for male development.

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I would like to thank the Pilgrim lab for many fond memories during my three years in the lab. David Pilgrim was an excellent supervisor during that time and I appreciate his patience and understanding over the years. Dave Hansen and Morris Maduro were a lot of fun to work with and I am glad to be able to call them my friends as well as my workmates. Ramesh Bhambani was also a good friend in the lab and always kept things interesting with his games and plans. I look forward to keeping in touch with all of these people well into the future. I would also like to thank Andrew Simmonds, Eugene Chomey, Rob Simpson and the rest of the department for making my stay an enjoyable one. Finally, I want to thank Angela, without whom I would have never survived these years. Meeting you in this lab made me never doubt that I was doing the right thing.

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Abbreviations

βGal	Beta-galactosidase
CEM	cephalic companion cells
DAPI	4,6-diamidino-2-phenylindole
depc	diethyl pyrocarbonate
DTC	distal tip cells
DTT	di-thio-threitol
gf	gain of function
HSN	hermaphrodite specific neurons
L1-L4	larval stages
lf	loss of function
m, z	maternal, zygotic
mcs	multi-cloning site
mx	mixed character
NLS	nuclear localization signal
PCI	phenol:chloroform:isoamyl alcohol
PCR	polymerase chain reaction
RACE	Rapid Amplification of cDNA ends
TE	tris, EDTA
ts	temperature sensitive
TSP	temperature sensitive period
UTR	untranslated region

INTRODUCTION

General

The goal of this thesis is to help to understand some of the mechanisms underlying signal transduction pathways. This study focuses directly on sex determination in *Caenorhabditis elegans* and the role that *fem-2* plays in the signal transduction pathway that determines sex. In order to better understand *fem-2*'s role, I have determined the temporal and spatial expression patterns of *fem-2*. This analysis allows a better understanding of how *fem-2* functions in determining sex and provides insight into general mechanisms of signal transduction pathways.

Caenorhabditis elegans is a model for studying a large number of biological processes as it is a multi-cellular eukaryote that is easy to study (Brenner, 1988). Although a simple organism, *C. elegans* appears to use many processes that are conserved throughout the animal kingdom. The two sexes, hermaphrodites and males, are composed of 959 and 1031 somatic nuclei, respectively (Sulston *et al.*, 1983). *C. elegans* are transparent throughout their development and consequently, the cell lineage of each individual somatic cell has been recorded using Nomarski microscopy and observed to be essentially invariant (Sulston *et al.*, 1983). The haploid genome of $8x10^7$ nucleotide pairs which has been mapped to 6 linkage groups is composed of 80% single copy sequences of which over 95% have been assembled into contiguous overlapping sequences (contigs) (Coulson *et al.*, 1991, Wilson *et al.*, 1994). This is approximately one half the size of the *Drosophila melanogaster* genome and efforts are currently underway to sequence the entire *C. elegans* genome. To date, this has been accomplished

for most of Linkage group III, the central region of Linkage group II and a small portion of the X chromosome (Sulston *et al.*, 1992, Wilson *et al.*, 1994).

C. elegans is a particularly popular organism for developmental biologists, especially those investigating the development of the nervous system, embryonic cell patterning, muscle development and the determination of sex. In sex determination, a simple signal (ratio of sex chromosomes to sets of autosomes) is capable of initiating a large number of developmental decisions that eventually affect up to 40% of the nematode's somatic cells (Hodgkin, 1988). Despite the fact that there are only three options for a cell, male, female or neuter, once the choice is made it sets in motion a vast number of interactions necessary for the final products. Recently, some of the genes involved in the determination of sex have been identified and the beginnings of a pathway for molecular regulation has been formulated (for reviews see Villeneuve and Meyer, 1990, Kuwabara and Kimble, 1992, Hodgkin, 1992). Evidence suggests that the early part of the pathway specific to sex determination involves trans-membrane signaling while later steps involve the control of transcription. Precisely how the trans-membrane signal is relayed to the transcription factor in the nucleus is not yet known but involves a number of proteins, one of which is FEM-2. Through its interactions with other sex determination proteins, FEM-2 is able to respond to the trans-membrane signal and regulate the transcription factor either directly or indirectly. This may involve a number of molecular mechanisms, including protein/protein interactions, post-transcriptional regulation and possibly kinase/phosphatase interactions. Determining precisely how this regulatory hierarchy will aid in the general understanding of how signal transduction pathways function.

Sexual Dimorphism

C. elegans, has a reproductive life cycle of approximately four days which begins as an egg, advances through four larval stages (L1 to L4) and ends with adults (Wood, 1988). The sexes are determined by the ratio of sex chromosomes to sets of autosomes (X/A ratio, Madl and Herman, 1979). Diploid animals with two X chromosomes (XX) are hermaphrodites while those with one X (XO) are males. Hermaphrodites produce both sperm and then oocytes and are self-fertile, producing a brood of self progeny consisting of almost entirely XX hermaphrodites (Hodgkin, 1988). Males arise at a frequency of approximately 0.5 % among these progeny due to meiotic X-chromosome non-disjunction (Hodgkin *et al.*, 1979). Hermaphrodites (XX) can also cross with males (XO), producing approximately equal numbers of male and hermaphrodite outcross progeny (Hodgkin, 1988). Unlike *C. elegans*, most species in the Phylum *Nematoda* are gonochoristic (having males and females) including the close relative of *C. elegans*, *C. remanai* (Hodgkin, 1988). Examination of *C. elegans* hermaphrodites and *C. remanai* females reveals no obvious somatic differences and suggests the only difference lies in the ability of the germline to produce sperm. This is supported by the observation that feminizing mutations such as *fem-2* have no obvious effects on the soma of XX animals (Kimble *et al.*, 1984). Taken together, this evidence suggests that hermaphrodism is a recent specialization of a previously male/female species (Hodgkin, 1988). I will therefore refer to *C. elegans* hermaphrodites as modified females that produce a limited number of sperm during the fourth larval stage before switching to oogenesis.

Of the somatic nuclei in the adult, 650 are sexually indifferent while the remainder (30% of hermaphrodite nuclei and 40% of male nuclei) are

dimorphic in that they have different fates in XX and XO animals (Hodgkin, 1988). Embryonic development is almost identical between the two sexes, the only lineage differences being two sets of programmed cell deaths; 4 male specific cephalic neurons (CEM) in hermaphrodites and two hermaphrodite specific motor-neurons (HSN) in males (Sulston and Horvitz, 1977). The early larval animal produced after embryogenesis (556 cells in XX animals, 558 in XO, Hodgkin *et al.*, 1989) then starts on one of two extensively different pathways towards becoming an adult male or hermaphrodite. Of the cells with a post-embryonic lineage, a small number are monomorphic resulting in 122 surviving nuclei (Hodgkin *et al*, 1989). The remaining cells with post-embryonic lineages adopt either the male or the hermaphrodite pathway. In addition, a number of other cells adopt similar lineages but perform different functions in the two sexes (e.g., intestinal cells). The germ cells also undergo different divisions in males and hermaphrodites (Kimble and Hirsh, 1979). Due to the complexity of the sexual dimorphism, anything more than a brief review of the sex-specific differences would be beyond the scope of this paper (see Hodgkin, 1988 and White, 1988 for more detail).

The germline is derived from two precursor cells, Z2 and Z3 in both XO and XX animals (Sulston and Horvitz, 1977). Mitotic proliferation occurs in the distal part of the gonad under the control of the distal tip cells (DTC) of the somatic gonad (Kimble and White, 1981). Meiosis begins in the proximal portions of the gonad (L3 in XO, L3/L4 molt in XX) with the first cells in both sexes differentiating into sperm. In the XX germline the differentiation then switches to oogenesis (Figure 2). The male can produce up to 4000 sperm in a lifetime and have been observed to produce up to 2871 progeny (Hodgkin, 1983b). An unmated hermaphrodite will continue

oogenesis until all of the stored sperm are used up (320 in total, Hodgkin, 1983b). Oogenesis then arrests unless stimulated by insemination by a male, eventually leading up to as many as 1400 total progeny (Hodgkin, 1988).

The primordial somatic gonad is identical in XO and XX L1 larvae, consisting of two cells, Z1 and Z4 (Kimble and Hirsh, 1979). In adult hermaphrodites the gonad includes a central uterus and two gonad arms, each containing a distal tip cell. The distal tip cells control germ cell differentiation and elongation of the gonad arms. In males, Z1 and Z4 differentiate into 56 nuclei consisting of one gonad arm, a vas deferens, a seminal vesicle, two distal tip cells and one linker cell (present in early development only). The distal tip cells control germ cell proliferation and the linker cell interacts with cells of the proctodoeum to open the pathway between the vas deferens and cloaca.

Some of the muscle cells differ between the sexes. In the hermaphrodite most of these muscles are involved in egg laying, including uterine and vulval muscle cells. In the male, most of the specific muscle cells are involved in copulation, especially tail curling (Sulston *et al.*, 1980). There are also various neuronal differences between the sexes. The only type of behavior unique to hermaphrodites is egg laying and there are 12 sexually specialized neurons involved in this (White *et al.*, 1986). The male has at least 87 specialized neurons most of which are involved in mating behaviour. In addition to these there are also the CEM neurons (cephalic companion cells) which are thought to be involved in chemotaxis of males towards hermaphrodites (Sulston and Horvitz, 1981). Unlike the hermaphrodite nervous system, the entire nervous system of the male has not been completely reconstructed (White *et al.*, 1986) therefore it is possible that many other neurons have a different circuitry in the two sexes. Other

sex specific structures are the vulva in hermaphrodites, the male tail and the intestine. Kimble and Sharrock (1983) demonstrated that yolk proteins are only made in the adult hermaphrodite intestine and are then secreted into the pseudocoelom and taken up by the gonad to be incorporated into the developing oocytes.

It is obvious that XX and XO newly hatched larvae undergo extensive differentiation in order to become adult males or hermaphrodites. XO development is relatively straightforward as its soma and germline are both masculine. The genes/products responsible for feminization are repressed throughout the development of the male and the genes/products responsible for masculinization are active at the appropriate times, early (embryo to L1) for somatic development and later (L1 onward) for spermatogenesis. XX development of the soma is opposite to that of the XO animal as the masculinizing genes are negatively regulated while the feminizing genes are activated. The modification of the germline to allow sperm production (producing hermaphrodites as opposed to females) presents the paradox of a male tissue (sperm) in a female somatic gonad. If *C. elegans* did at one time exist as a male/female species, in order to evolve into a male/hermaphrodite species it must have developed a mechanism capable of modulating the genes or their products necessary for oogenesis. This modulation must not affect female somatic development (either by being too late or by being restricted to the germline tissue) and must be transient so that oogenesis can begin afterwards.

Interpreting the X/A ratio

Organisms that use the X/A ratio to determine sex face two problems. The first is how to quantify the ratio into a molecular signal. The second is

how to compensate for the differences in dosage for X-linked genes. Over the past twenty years genetic analysis of *C. elegans* dosage compensation and sex determination mutants have uncovered an epistatic pathway. This pathway is branched and involves genes specific to one process such as the genes specifically involved in dosage compensation and those specifically involved in sex determination such as *fem-2* as well as a number of genes involved in the regulation of both (Figure 3). For this thesis, the genes specific to dosage compensation will be largely ignored. The genes involved in interpreting the X/A ratio and thereby activating and/or repressing the sex determination and dosage compensation branches of the pathway are however important for understanding sex determination.

How the X/A ratio triggers the choice of sexual fate is not entirely understood. Three regions of the X chromosome have been identified that are thought to contain sex determination signal elements (Akerib and Meyer, 1994). Four genes are involved in transmitting the signal to the separate branches for sex determination and dosage compensation. These genes include *xol-1* (XO-lethal) and *sdc-1,2* and *3* (sex determination and dosage compensation, Rhind *et al.*, 1995). Mutations in *xol-1* cause the feminization of XO animals as well as lethality attributed to improper dosage compensation (Miller *et al.*, 1988). In *C. elegans*, dosage compensation is achieved through reducing the expression of the X chromosome in XX animals via the *dpy*-21 (dumpy body shape due to no dosage compensation), *dpy-26, dpy-27, dpy-28* and *dpy-30* genes (reviewed by Kuwabara and Kimble, 1992). In *xol-1* mutants, the *dpy* genes are improperly activated resulting in the genes on the single X chromosome not being expressed at high enough levels (Miller *et al.*, 1988). Epistatic interactions suggest that *xol-1* acts before the *sdc* genes. The three *sdc* genes

have the opposite role of *xol-1* in that they are required for hermaphrodite development (Meneely, 1990). Mutations in any of these genes results in masculinization and lethality of XX animals. The low viability seems to be due to abnormally high X-linked gene expression and as expected, XO animals are unaffected. It has been hypothesized that *xol-1* responds to the X/A ratio (directly or indirectly) and acts by turning off the *sdc* genes (if X/A ratio is low). The role of the *sdc* genes is both to turn on the dosage compensation *dpy* genes and suppress the activity of the first gene in the sex determination branch, *her-1* (hermaphrodite), which causes XX and XO animals to develop into hermaphrodites (XO animals are feminized somatically and produce both sperm and oocytes in their germline). Therefore, in XO animals, *xol-1* will suppress the *sdc* genes, preventing the activation of the dosage compensation pathway and allowing the sex determination pathway to proceed with *her-1* activity which promotes male development. In XX animals, *xol-1* activity is low, allowing the *sdc* genes to activate the dosage compensation genes to down regulate X chromosome expression.

The Sex Determination Branch

There have also been mutations isolated in genes specifically involved in sex determination. The first class of mutations isolated were those that masculinized XX animals (transformer genes; *tra-1, tra-2* and *tra-3*; Klass, *et al.*, 1976, Hodgkin and Brenner, 1977, Klass *et al.*, 1979). All of the *tra-2* and *tra-3* loss of function alleles (lf) masculinize both the soma and the germline (by causing sperm production) indicating that they are necessary for wildtype female development in XX animals. *tra-1 (lf)* alleles also masculinize the soma of XX animals but, feminize the germline of XX and

XO animals, indicating that *tra-1* plays different roles in the germline and the soma (Hodgkin, 1986, Hodgkin, 1987, Schedl *et al.*, 1989). Analysis of the interactions between these mutants and *her-1* led to a pathway (Hodgkin, 1980, Doniach and Hodgkin, 1984). This model proposed that the X/A ratio (through *xol-1* and the *sdcs*) determines the activity of *her-1* (*her-1* activity is low in XX animals) which in turn negatively controls the activity of *tra-2* and *tra-3*. *tra-2* and *tra-3* activity then control *tra-1* which is on for hermaphrodite development and off for male development. Another class of mutations had the opposite effect from the *tra* genes. These loss of function mutants (*fem-1, fem-2, fem-3*) feminize the germline of XX and XO animals and the soma of XO animals, with the strongest alleles resulting in fertile females (Nelson *et al.*, 1978, Kimble *et al.*, 1984, Hodgkin, 1986). This indicates that the wildtype function of the *fem* genes is to promote male germline and somatic. Identification of the *fem* genes and the role of *tra-1* in the germline led to a revised model. The *fem* genes were placed between *tra-2/tra-3* and *tra-1* in the soma resulting in a cascade of negative regulatory interactions of on/off switches ending with *tra-1* as the terminal regulator in the soma (Doniach and Hodgkin, 1984, Hodgkin, 1986). The germline model was reorganized by placing the *fems* as the terminal regulators. The role of *tra-1* in the germline remains obscure. A number of other genes have since been identified and placed in the pathway resulting in a complex model including genes involved in somatic or germline sex determination and some common to both decisions (Figure 3; for a review see Hodgkin, 1990, Villeneuve and Meyer, 1990, Kuwabara and Kimble, 1992).

Focus then turned to the molecular mechanisms underlying this pathway. Of particular interest to the members of our laboratory was *fem-2*

and its interactions with the other *fem* genes. The dual role of the *fem* genes as negative regulators of *tra-1* in the soma and terminal regulators of spermatogenesis in the germline suggest that they may have complex interactions with a number of other sex determination genes. In order to fully understand these interactions with the other genes it is necessary to discuss in detail the previously mentioned genes as well as a number of additional ones.

her-1

her-1 (her maphrodite) is required for male development. *her-1 (lf)* mutations result in XO animals developing as hermaphrodites (Hodgkin, 1980) and gain of function (gf) mutations partially masculinize XX animals (Trent *et al.*, 1988). The epistatic pathway reveals that *her-1* negatively regulates *tra-2* in order to promote male development. The predicted protein sequence of HER-1 suggests that it is an extra-cellular secreted protein of low molecular weight which may negatively regulate TRA-2 directly by binding to it (Figure 4, Perry *et al.*, 1993). This is supported by the observation that mosaic analysis suggests that *her-1* is involved in cell interactions in sex determination (Hunter and Wood, 1992).

tra2/tra-3

TRA-2 and TRA-3 seem to promote female development in the germline and the soma by suppressing the activity of the *fem* genes, which are responsible for male somatic development and sperm production. The nature of this interaction is not yet clear but some recent findings are helping us to understand it somewhat. *tra-3* has been cloned (T. Barnes, personal communication) and the predicted protein sequence resembles

calpain, a calcium-regulated, cytosolic protease found in a wide variety of species. Genetic analysis suggests that it may act as a positive regulator of TRA-2 and a possible model proposes that it may cleave TRA-2 in order to activate it.

The molecular characterization of *tra-2* suggests that the mRNA encodes a large membrane protein which is probably the receptor for HER-1 (Figure 4, Kuwabara *et al.*, 1992). Three classes of *tra-2* mutations have been identified resulting in different phenotypes in XX and XO animals as well as in the germline and the soma. Loss of function mutations cause incomplete masculinization of XX animals and most likely are unable to negatively regulate the *fem* genes (Klass *et al.*, 1976, Hodgkin and Brenner, 1977, Hodgkin, 1980). Most of the loss of function mutants affect the cytosolic, carboxy terminus and this is probably the region responsible for negatively regulating the *fems*. Recent evidence suggests that TRA-2 may interact directly with FEM-3 (A. Spence, personal communication). There have also been two other types of *tra-2* mutants discovered; dominant gain of function mutants (gf) and mixed character mutants (mx) (Doniach, 1986, Schedl and Kimble, 1988, Okkema and Kimble, 1991, Goodwin *et al.*, 1993). *tra-2 (gf)* mutants result in feminization of XX animals and some feminization of XO germline and intestines (Goodwin *et al.*, 1993). These mutants map to a perfect direct repeat in the 3' UTR (untranslated region) of the *tra-2* mRNA which seems to be necessary for one type of negative regulation of *tra-2*. Recent evidence suggest that the protein responsible for this is LAF-1 (lethal and fog [feminization of the germline]), which causes feminization of the germline and larval lethal phenotypes (J. Kimble, personal communication). The third type of *tra-2* mutants (*tra-2mx*) map to the carboxy terminus of TRA-2 and may interfere with negative regulation by

FOG-2. *fog-2* mutations feminize the germline but not the soma of XX animals and are thought to negatively regulate TRA-2 in a tissue-specific manner. At the same time, the *tra-2 (mx)* mutations may also interfere with TRA-2's ability to negatively regulate the *fems*, hence the mixed phenotype of loss and gain of functions (J. Kimble, personal communication). It therefore seems that TRA-2 is an important protein that negatively regulates the *fem* genes possibly through binding FEM-3 while itself being negatively regulated by at least three different proteins. LAF-1 and HER-1 are probably responsible for preventing TRA-2 from negatively regulating the *fems* in XO animals and FOG-2 most likely is responsible for modulating germline TRA-2 in XX animals in order to allow the *fems* to produce the short period of spermatogenesis.

The *fem* genes

Loss of function mutations (probable nulls) in each of the *fem* genes show essentially the same phenotype; complete feminization of XX and XO animals at 25°C (Hodgkin, 1986). Epistasis studies suggest that all three act at the same point in the pathway in order to promote spermatogenesis in the germline and male somatic development in XO animals by negatively regulating TRA-1. How these genes function in the germline and in the soma is not yet known. All three mutants can be maternally rescued (*fem/fem* mutants from *fem/+* mothers are not completely feminized) to some degree (except for the *fem-3* XX germline genotype) but there are subtle differences between them (Doniach and Hodgkin, 1984, Kimble *et al.*, 1984, Hodgkin, 1986). For example, *fem-3* mutants show haplo-insufficiency for the XX germline phenotype (Hodgkin, 1986). Heterozygous *fem-3* animals are feminized and mutants with heterozygous mothers (m+z-;

m=maternal genotype and z=zygotic genotype) are not rescued. All three mutations also show some degree of zygotic rescue (m-z+), once again with some differences between the three (for example, the XX germline phenotype of *fem-3* cannot be zygotically rescued). These differences may only represent how stringent the requirements are for each gene product or it may represent differences in the nature of their actions.

fem-1 has been cloned and shown to encode a soluble, intracellular protein that contains six copies of a *cdc10/SWI6* repeat (also called ANK repeat) near its N-terminus (Spence *et al.*, 1990). Other proteins containing this motif have been implicated in protein/protein interactions and the motif may be important for binding, but the target is unknown (Figure 4). *fem-3* has also been cloned and found to encode a novel, soluble protein (Figure 4; Rosenquist and Kimble, 1988). *fem-3* mRNA contains an inverted repeat in the 5'UTR as well as a regulatory element in the 3' UTR (Ahringer and Kimble, 1991, Ahringer *et al.*, 1992). The 3'UTR has been shown to be necessary for the post-transcriptional, negative regulation of *fem-3*, possibly by determining the length of its poly(A) tail (Ahringer *et al.*, 1992). *fem-3* gain of function mutations have been mapped to the 3'UTR and presumably prevent binding of a repressor protein (Ahringer and Kimble, 1991). These mutations cause masculinization of the XX germline with no apparent effect on the soma (Barton *et al.*, 1987). The repressor protein may be the product of one of the *mog* genes (masculinization of the germline, J. Kimble, personal communication). Apparently, this form of repression is restricted to the germline of XX animals and the temperature sensitive period of *fem-3 (gf)* mutation suggests that it occurs late in development (L4 to adult, Barton *et al.*, 1987). Early in development TRA-2 may negatively regulate the *fems* in XX animals (to produce female soma) but

allows them to be transiently active in the germline so sperm can be formed. It may be that once TRA-2 has been turned off another mechanism must be invoked to turn the *fems* off in order to allow oogenesis to proceed. This may be done by one of the *mog* gene products and *fem-3 (gf)* mutants may be insensitive to this late repression.

Two lines of evidence help to make predictions about how the *fem* genes interact. The first involves double mutant analysis of temperature sensitive alleles (ts) which retain partial function at restrictive temperatures. Single mutants of any of the three *fems* (ts alleles) show incomplete feminization at 25°C but double and triple mutants show complete feminization (Kimble *et al.*, 1984, Hodgkin, 1986). In addition, the incomplete zygotic rescue of the *fem-3* XX germline phenotype can be exacerbated by *fem-1* or *fem-2* mutations. The second line of evidence involves double mutant analysis of the *fem* genes with *fem-3(gf)* mutants. *fem-3* gain of function mutations can suppress the effects of *fem-1* and *fem-2(ts)* alleles. They can also aid the maternal rescue of *fem-1/+* mothers of *fem-1/fem-1* daughters (m+z-). This information suggests that the products of the three *fem* genes directly interact perhaps as a multi-protein complex (Kuwabara and Kimble, 1992). This is supported by the observation that the temperature sensitive period (TSP) of all three genes overlap (see later section on TSP). In this model, the complex may be regulated directly through *fem-3* while *fem-1* and *fem-2* act to either promote spermatogenesis or repress the actions of TRA-1. FEM-2 has protein phosphatase activity (Pilgrim *et al.*, 1995; D. Hansen, personal communication) but its function in this putative complex is not yet known. FEM-3, FEM-1 and TRA-1 all have putative phosphorylation sites and could be targets for FEM-2.

An alternative model is that the activity of the *fem* genes is controlled by directly repressing FEM-3 activity. Active FEM-3 is able to activate FEM-2, which in turn dephosphorylates FEM-1, which now negatively regulates TRA-1 (Figure 4). *fem-3 (gf)* mutations would result in higher levels of FEM-2 and FEM-1 which may explain how it is able to partially suppress the effects of *fem-1* and *fem-2* temperature sensitive mutations. If the (ts) mutants retain only partial function then increasing their levels in the cell might compensate for their impaired function. This is supported by the observation that *fem-3 (gf)* mutations do not suppress the effects of *fem-1* null mutants (Barton *et al.*, 1987).

tra-1

tra-1 (Hodgkin and Brenner, 1977) mutations have a similar phenotype to *tra-2* and *tra-3*, but with a number of notable exceptions. XX animals in which *tra-1* activity is absent develop a male non-gonadal soma, regardless of whether other sex determining genes are functional (Hodgkin, 1980, Hodgkin, 1986). The *tra-1* gene expresses two mRNAs via alternative splicing that encode two related zinc finger proteins (Zarkower and Hodgkin, 1992), only one of which binds DNA *in vitro* (Zarkower and Hodgkin, 1993). It therefore seems that TRA-1 is the terminal regulator in the soma, either activating or repressing the transcription of sex specific genes in XX animals (Figure 4). This activity in the soma is cell-autonomous, consistent with the role of a transcription factor (Hunter and Wood, 1990). This activity is negatively regulated by the *fem* genes in XO animals in an unknown manner. The role of *tra-1* in the germline is more complicated. *tra-1* null mutants lead to a male germline in XX and XO animals which produce reduced sperm and occasionally contain oocyte-like

cells (Hodgkin, 1987, Schedl *et al.*, 1989). This feminization can also include the gonadal soma and is quite variable, depending on the allele. This suggests that in addition to TRA-1's female-specific activity in the non-gonadal soma, it plays a role in germline spermatogenesis as well. Perhaps TRA-1 plays completely opposite roles in the germline and the soma, a somewhat counterintuitive but nonetheless, precedented role (Baker and Belote, 1983). Alternatively, the putative loss of function mutants may create novel germline products which result in feminization (Schedl *et al.*, 1989). This may be possible due to the multiple products and auto-regulation of *tra-1* but does not explain the recessive nature of the feminization.

Gain of function *tra-1* mutations have also been isolated (Hodgkin, 1980, Hodgkin, 1983a, Hodgkin, 1987, Schedl *et al.*, 1989). These feminize both the soma and the germline, supporting TRA-1's role in the non-gonadal soma but further complicating the germline role. Analysis of double mutants has shown that *tra-1 (lf)* germline feminization is epistatic to all known masculinizing mutants in the germline (including *fem-3 (gf)*) but the sperm produced is dependent on the *fem* genes and *fog-1* (Schedl *et al.*, 1989, Hodgkin, 1987, Barton and Kimble, 1990), demonstrating that they may be the terminal regulators in the germline. TRA-1's role in the germline therefore remains in question (Spence *et al.*, 1990, Zarkower *et al.*, 1994). Recent experiments have mapped the gain of function mutants to a small region of sequence present in both proteins (de Bono *et al.*, 1995). This site contains a putative phosphorylation site and may represent a region necessary for protein/protein regulation.

Other genes

In addition to the "core" genes mentioned above, there are a number of other genes that are involved in the regulation sex determination but have not been well characterized. These include *fog-1,-2* and *-3, laf-1* and *mog -1 to -6,* some of which have been briefly mentioned above (Figure 3). These genes were not used in the experiments described here but play a minor role in some of the proposed models in the discussion.

fem-2

The *fem-2* locus maps at the left end of linkage group III (Pilgrim, 1993). There are four known alleles, two of which are temperature sensitive (*q117* and *b245*) and two which are putative null alleles (*b245e2005* and *e2102* [the strongest allele discussed by Hodgkin (1986), *e2105* has recently been shown to be an accidental re-isolation of an earlier allele *b245e2005*, Pilgrim *et al.*, 1995]). *q117* and *b245* mutants are incompletely feminized even at the restrictive temperature (Kimble *et al.*, 1984). *b245e2005* and *e2102* mutants show complete feminization at 25°C (Hodgkin, 1986), while at 20°C, XO animals show an incompletely feminized tail. Molecular analysis revealed that both are nonsense mutants (Pilgrim *et al.*, 1995). This would imply that male somatic development may not require *fem-2* activity at lower temperatures. The protein sequence of FEM-2 shows similarities with protein phosphatases of the type 2C (Pilgrim *et al.*, 1995). cDNA analysis is consistent with a single transcript from the region (although the cDNA library was made from XX RNA) and this cDNA attached to the *fem-2* upstream region (the final construct includes the first *fem-2* intron) has been shown to be capable of rescuing all *fem-2* mutant phenotypes (Pilgrim

et al., 1995). This suggests that only one transcript is produced from the *fem-2* locus.

Maternal Rescue

The complete *fem-2* mutant phenotype is only seen in *fem-2/fem-2* daughters of *fem-2/fem-2* mothers (m-z-). Homozygous *fem-2* daughters of a *fem-2/+* mother (m+z-) are not completely feminized, indicating maternal rescue of some of the phenotypes. XX (m+z-) animals are still hermaphrodites but have smaller self-brood sizes indicating a reduction in sperm production (Hodgkin, 1986). XO animals are only completely feminized in the condition m-z- and in addition, only when the progeny are raised at 25°C. At 20°C the XO animals (m-z-) are intersex and at 15°C only partially feminized. XO animals (m+z-) can be partially rescued by maternal product, once again in a temperature sensitive fashion (at 20°C XO animals are fertile but produce less sperm and at 25°C they are infertile). Maternal product can rescue the male somatic phenotype completely but only partially rescue the germline. Therefore, for full male development it is probable that zygotic product is required at this point. This is supported by the observation that crossing *fem-2/fem-2* females with wildtype males (m-z+) results in fertile hermaphrodites (zygotic rescue). The rescued hermaphrodites have approximately wildtype brood sizes suggesting that the rescue is complete. To complicate matters though, it also seems that the male somatic phenotype can be completely zygotically rescued. This suggests that male somatic development can be controlled by either maternal or zygotic product.

Temperature sensitive periods

In order to determine when *fem-2* product is necessary during development, Kimble *et al.* (1984) analyzed a temperature sensitive mutant (*b245*). By shifting animals either down from 25°C (restrictive) to 20°C (permissive) or by shifting them up it is possible to determine when the temperature sensitive period is (TSP, measured at 25°C, hatch time=0). In the simplest case, the TSP represents the period of synthesis and/or function of *fem-2*. Brood sizes of shifted animals were counted and assumed to be indicative of the amount of sperm produced by the hermaphrodite (sperm is the limiting factor in self-brood sizes). The TSP for the germline in XX animals was found to be between L1 and L2 (8-20 hours). In XO animals, the phenotype was measured by scoring the number of XO animals that did not have oocytes in their germline. The TSP for the germline of XO animals was observed to begin in L2 and extends into adulthood (15 hours and onward). The TSP for gonadal-somatic tissue (e.g. formation of vulva, yolk proteins and gonad) in XO animals was found to be 0-12 hours after hatching. *b245* animals are not completely feminized at 25°C (XO animals are completely feminized except for their tails) therefore the TSP could not be determined for the non-gonadal soma. Kimble *et al.* (1984) observed that *fem-1(ts);fem-2(ts)* double mutants are completely feminized at 25°C (even though neither are individually) and determined that the TSP for normal development of the XO tail for this double mutant was a broad period spanning the first two larval stages (4-20 hours). All of the above TSPs were determined using *b245* daughters of *b245* mothers (m-z-) therefore not distinguishing whether the TSPs are for zygotic or maternal product.

TSPs have also been determined for some of the phenotypes of *fem-1* and *fem-3* and were found to overlap that of *fem-2* (Nelson *et al.*, 1978,

Hodgkin, 1986). The TSP was also determined for *fem-3 (gf)* mutations which cause XX animals to make only sperm, with no effects on somatic tissue and was found to extend from mid-L4 until early adulthood (35-42 hours) (Barton *et al.*, 1987). From these results it is apparent that the time at which the decision is made to develop as a male or female varies according to the tissue. For example, the TSP for the somatic tissues appears to be early in development (embryo to L2) which coincides with the first differences in the lineage between the two sexes (Kimble and Hirsh, 1979). The TSP for germline development occurs later (L2 to adult depending on sex) which correlates well with the onset of germ cells into meiosis (Kimble and Ward, 1988). Therefore, there is an early decision concerning somatic development followed by later, ongoing decisions to maintain spermatogenesis in XO animals or switch to oogenesis in XX animals. The phenotype and TSPs of *fem-2* clearly indicate that it is involved in both of these decisions. It is unclear how maternal and zygotic product interact in each decision and the nature of these product's interactions with the other sex determination genes.

Statement of goals

The goal of this project was to examine the spatial and temporal expression patterns of *fem-2*. Two different approaches were taken. The first step involved examining the expression levels of *fem-2* throughout development of XX and XO animals using standard Northern analysis. The second approach was to examine *fem-2* expression patterns using a construct consisting of the *fem-2* regulatory region driving an easily assayed 'reporter gene'. Transgenic animals carrying this should show expression under the control of the *fem-2* promoter. Once the expression patterns were well

known, they could be examined in a number of mutant backgrounds. Any observable changes in the expression patterns could then provide some insight into the regulation of *fem-2* expression. Comparison of the above information with the previously determined TSPs of the various *fem-2* phenotypes should allow a number of predictions on where, when and how *fem-2* is working. This would provide the basis for future experiments to test some of the models.

MATERIALS AND METHODS

C. elegans strains and growth conditions

A list of *C. elegans* strains used in this study may be found in Table 1. Stocks of *C. elegans* were maintained and manipulated according to Brenner (1974) at 20°C unless otherwise mentioned. For anatomical studies, live animals were examined by anaesthetizing small numbers in 5 µl of 1% phenoxy-propanol on 2% agar pads (Sulston and Hodgkin, 1988). Animals were then examined using a Zeiss Axioskop microscope equipped with Differential Interference Contrast (DIC) optics and an attached halogen lamp for incident-light fluorescence. Photographs were done using a mounted MC 80 Microscope camera (Zeiss) and Kodak technical pan film, iso 25/15°. The prints were done using either Kodak polycontrast RC glossy paper or Ilford multigrade IV, RC deluxe, glossy paper as described by the manufacturer.

Strains carrying transgenes were classified as either those carrying extra-chromosomal arrays (*edEx*) or integrated sequences (*edIs*). An extra-chromosomal array is a large piece of DNA containing a number of copies of the plasmids injected into the oocytes of the P1 generation. The plasmids are thought to be assembled into arrays through homologous recombination (Mello *et al.*, 1991). Integrated sequences are a portion of an array inserted into the genome at a double strand break induced by gamma irradiation.

Microinjection of Nematodes

Micro-injections were performed essentially as described in Mello *et al.*, (1991) with the following modifications. The experiments used a

mixture of the pRF4 plasmid containing the dominant *rol-6* marker [*rol-6(su1006dm)*] and the test plasmid in a 1:1 ratio. Prior to injection, young well-fed adult hermaphrodites were transferred to plates containing no bacterial food source. Five to fifteen worms were then immobilized on a dried pad of 1.2% agarose under a 2 µl drop of liquid paraffin oil (BDH). Agarose pads were prepared using 22 X 40 mm glass cover slips. The microscope used for injection was a Zeiss Axiovert 10 equipped with Differential Interference Contrast optics. After all the worms on a pad were injected, 25 µl of M9 buffer (see Appendix) were added to the injection pad. After 15 minutes the nematodes were transferred to a seeded (containing a bacterial food source) agar plate and the worms were allowed to recover overnight at 20°C. Surviving animals were then transferred to individual plates and their progeny examined for the dominant rolling phenotype. Some animals were tested for the presence of the test plasmid using single worm PCR. Rolling animals that transmitted the transgenic array to their progeny were either examined directly or mutagenized in order to obtain an integrated line. The latter was achieved by exposing young adult, transgenics to 3800 rads of gamma rays (using a Co^{63} source) and screening for F2 transgenics that gave 100% rolling progeny. Transgenic strains such as DP98 which are self-sterile were crossed into a suppressing background [*fem-1(hc17);him-8(e1489)*] and then mutagenized.

Mutant strains crossed with DP90 (*edEx26*[pDP#TJ01;pRF4]) in order to examine their effects on its staining patterns were: CB3347 [*fem-1(hc17ts); him-8(e1498)*], CB4002 [*fem-3(q20gf,sd,ts); dpy-20(e1282ts)*], CB3366 [*tra-2(b202ts)*], CB3807 [*tra-2(e1940dm,mx); vab-9(e1744)*], CB4108 [*fog-2(q71)*], CB3538 [*fem-2(b245ts); him-8(e1489)*] and DP71 [*fem-2(q117ts); him-8(e1489)*]. Sex determination mutants can be difficult to propagate as self-fertilizing

homozygotes because many result in sterile populations. For example, *fem-2* homozygous null mutants are self-sterile. This problem was overcome by carefully choosing alleles that allow for the easy maintenance of the strains. For the mutants *fem-1(hc17)*, *fem-3(q20gf)*, *tra-2(b202)* and *fem-2(b245* and *q117)* this was achieved by using temperature sensitive alleles that are fertile at permissive temperatures. There are however, no temperature sensitive alleles for *fog-2* or for *tra-2* mixed character alleles. Fortunately, both of the alleles chosen for these genes have mutant effects that are specific for only one sex, thereby allowing the maintenance of a male/female strain. For example, *fog-2(q71)* mutations only feminize XX animals which allows the strain to be maintained by outcrossing the feminized XX animals (females) with the unaffected males. *tra-2(e1940mx)* alleles are similar; they result in complete feminization of XX animals and mixed transformation of XO animals that remain fertile males.

The crosses were further complicated by the fact that the extra-chromosomal array (arrays carrying the transgenes) was phenotypically marked with the dominant *rol-6* (roller) marker and some of the arrays also caused a feminization of the germline (FOG) phenotype. XO animals carrying the array were rolling, partially feminized males that mated very poorly. In the construction of the strains the mutant allele was therefore always introduced by crossing heterozygous or homozygous males with females carrying the array. The resulting strain was maintained as a male/female strain by laborious backcrossing.

Some of the mutants also had a FOG phenotype, namely *fog-2(q71)* and *tra-2(e1940mx)*. Therefore, in order to identify mutants carrying the array, either a linked marker was used or siblings that did not carry the extra-chromosomal array were analyzed. *fem-3(q20gf)* and *tra-2(e1940mx)*

used the closely linked marker genes, *dpy-20(e1282)* and *vab-9(e1744)*, (variable abnormal) respectively. Mutants carrying the array were then identified by selecting animals displaying both the genetic marker and the *rol-6* phenotypes. *fem-1(hc17)*, *fog-2(q71)* and *fem-2(b245)* were not linked to useful markers and therefore a different selection technique was required. *fem-1(hc17); edEx26*, *fog-2(q71); edEx26* and *fem-2(b245); edEx26* animals were identified by picking putative mutants carrying arrays and raising a portion of their progeny at the restrictive temperature and the remainder at the permissive temperature. The progeny raised at the restrictive temperature that did not carry the array were then examined for the mutant phenotype. If all animals not carrying the array displayed the phenotype then it was assumed that the mother had been homozygous for the mutation and therefore the progeny that were raised at the permissive temperature were as well. Transgenic worms from the permissive temperature were then chosen and maintained.

A *tra-2(b202); edEx26* mutant strain was not stably established even after following the above selection procedure. It may be possible that this strain was not identified because it was infertile at the permissive temperature (there was no way to identify these mutants carrying the array at 15°C, as *tra-2(b202)* has no phenotype at this temperature). Worms with the genotype *tra-2(b202)/+; edEx26* were isolated that produced 25% *tra-2(b202)*; self-progeny when they were raised at 25°C. Unfortunately, these were sterile pseudomales at this temperature and could not be used for maintaining the strain. It was observed that one copy of *tra-2(b202)* could suppress the FOG phenotype caused by *edEx26* (see later results), even at the permissive temperature. It is possible that *tra-2(b202); edEx26* worms raised at the permissive temperature were sterile, or possibly lethal, explaining

why only heterozygotes of *tra-2(b202)* were isolated. Since one copy of *tra-2(b202)* suppressed the FOG phenotype of *edEx26* (see later results), heterozygotes could then be stably maintained on plates without males (which would select against self-sterile *edEx26* animals not carrying *tra-2(b202)*). Homozygous *tra-2(b202)* animals suitable for staining could then be obtained by shifting animals to 25°C and selecting F1 worms that were masculinized. Unfortunately, this did not allow the examination of staining patterns in younger worms. *fem-2(q117)/+; edEx26* worms were also created but *fem-2(q117)/fem-2(q117); edEx26* animals were not.

Molecular Biology Techniques

Standard methods (Sambrook *et al.*, 1989) were used for all molecular biology except where indicated. A list of plasmid vectors may be found in Table 2. Plasmid DNA was isolated with the following modifications. Preparations of bacteria were scaled up to 10 or 100 ml as necessary. The precipitation step was modified by using an equal volume of isopropanol, followed by phenol, phenol/chloroform and chloroform extractions followed by a final ethanol precipitation. DNA sequencing used the Sequenase 2.0 DNA sequencing kit as described by the manufacturer (USB) and was analyzed on 6% (38:2, acrylamide:bisacrylamide) polyacrylamide gels.

PCR

PCR was performed as described in Williams *et al.*, (1992) with the following modifications. Adult hermaphrodites were individually picked off plates and transferred to 2.5 µl of worm lysis buffer (see Appendix) in separate 400 µl microfuge tubes. The tubes were then frozen at -70°C for 10

minutes. A single drop of mineral oil was added to each tube and the tubes were heated to 65°C for one hour followed by 95°C for 15 minutes. A PCR mix (25 picomoles of each primer, 2.5 µl of 10X PCR reaction buffer (see Appendix), 1.25 mmoles of each dNTP, 0.5 µl of Taq polymerase (5 Units/µl) and H_2O to 25 µl) was spun down through the oil droplet. Amplification used the following parameters; one cycle of 95°C (5 minutes), 61°C (90 seconds), 73°C (3 minutes) and 29 cycles of 93°C (75 seconds), 61°C (75 seconds) and 73°C (135 seconds). The PCR machine used was a Robocycler 40 (Stratagene). The strains used were; DP98, non-rolling progeny of DP98, DP103, DP105, DP106 and DP107. Plasmid DNA was used as a positive control. The primers used were TGJ01 (5' AACCACTACCGGC TTCGGTGGGACAGCTGG 3'), and Reverse primer (5' AAACAGCTATGA CCAT 3').

Radio-labeling of DNA and RNA

DNA probes (50 pg to 1 µg) were radio-labeled using the T7 Quickprime kit (Pharmacia) as described by the manufacturer. The reactions were then added to 85 µl of TE plus 0.1% sarkosyl and pipetted onto a pre-packed, Sephadex column. The columns were made by adding sephadex in TE plus 0.1% sarkosyl to 1 cm^3 syringes with the plunger removed (which were blocked with glass wool) and centrifuged for 90 seconds at 4000 rpm in an IEC clinical centrifuge. The samples were centrifuged as above into microfuge tubes and 1 µl was aliquoted for scintillation counting.

RNA anti-sense probes were made using *in vitro* transcription. DNA templates were linearized using appropriate restriction enzymes and purified from low melting point agarose gels. Linearized template (0.1 to 1 µg) was mixed with 4 µl of 5X transcription buffer (see Appendix), 2 µl of 100

mM DTT, 40 U of RNase inhibitor, 10 mmoles each of CTP, GTP and ATP, 50 µCi of (α-^{32}P)-UTP, 20 U of T3 or T7 RNA polymerase and H_2O to 20 µl. Samples were incubated for 45 minutes at 37°C and the template DNA digested using 20 U of DNAse I (37°C for 15 minutes). Unincorporated nucleotides were removed by adding the sample to a 10X volume of Biogel P30 slurry which was then added to a 1.5 ml microfuge tube with a small hole poked in the bottom blocked by glass beads. The tubes were centrifuged for 2 minutes at 4000 rpm in an IEC clinical centrifuge and the sample collected. Samples were then heated at 95°C for 2 minutes, quenched on ice and added directly to hybridization solution (see Appendix).

Bulk growth of nematodes and stage isolations

Bulk populations of N2 worms and *him-8 (e1498)* worms were grown as described in Sulston and Hodgkin, (1988). Worms were frozen in liquid nitrogen and stored at -70°C until needed. Synchronized populations of worms were achieved using hypo-chlorite treatment of bulk worms containing large numbers of gravid adults. The purified eggs from this procedure were either frozen away or allowed to hatch overnight and then fed *E. coli* strain OP50. The worms were allowed to mature until the desired age, purified and then frozen for later use.

RNA isolation and poly(A) selection

RNA was isolated using the following procedure. For small quantities of nematodes the worms were washed off seeded plates (one 5 cm plate to four 9 cm plates) with M9 buffer and transferred to a microfuge tube. The worms were pelleted in a microfuge and the pellet resuspended in 400 µl of Guest buffer (see Appendix) and 400 µl of phenol: chloroform: iso-

amyl alcohol (PCI, 25:24:1). Sterile glass beads (0.5 grams, 0.3-0.4 mm diameter) were added and the tubes vortexed for two minutes. The liquid was then drawn off into clean microfuge tubes and the beads rinsed with 200 µl of Guest buffer and 200 µl of PCI. This was added to the previous liquid and the tubes were centrifuged for 2 minutes at 14000 rpm. The aqueous layer was transferred to a fresh tube and re-extracted with an equal volume of PCI. The nucleic acids were then precipitated with 1/10th volume of 3M Na acetate (pH 6.0) and two volumes of ethanol for at least 20 minutes at -20°C.

The tubes were centrifuged for 10 minutes and the pellets washed with 80% ethanol, then allowed to dry on the benchtop. The pellet was resuspended in 300 µl of $_{depc}H_2O$ and the RNA precipitated with 900 µl of 4M Na acetate (pH 6) for at least 4 hours at 4°C. The RNA was pelleted as above and the pellet resuspended in 100 µl of $_{depc}H_2O$ and precipitated again with 5µl of 3M Na acetate and 210 µl of ethanol for at least 20 minutes at 20°C. The RNA was pelleted as above and resuspended in 50 µl of $_{depc}H_2O$. RNA concentration and purity was estimated using spectrophotometric readings. Protein was often removed by additional phenol, phenol/chloroform and chloroform extractions.

This procedure was also used for small quantities (< 0.4 grams) of frozen worms. Larger preparations of frozen worms (0.5 to 2 grams) used a scaled-up version of the above protocol with the following modifications. Frozen worms were thawed enough that the frozen plug of worms could be removed into 50 ml plastic tubes containing 4 mls of Guest buffer, 4 mls of PCI and 12 grams of glass beads. The liquid was transferred to 15 ml tubes (Falcon #2059) along with the 1 ml of Guest/PCI used to wash the tubes. The rest of the protocol was then followed using ten times the volumes.

The above procedures are not efficient for isolating RNA from eggs. Any populations with eggs (or gravid hermaphrodites) were frozen at -80°C, transferred to a mortar and ground using liquid nitrogen. The ground worms were then used as starting material for the rest of the protocol.

Poly(A) mRNA was selected from total RNA using the polyATtract mRNA isolation (Promega) as described by the manufacturer.

Northern Blots

For buffer and hybridization recipes, see appendix. Five to twenty-five µg of RNA [total or poly (A)] in a volume of 1 to 10 µl was mixed with 10 µl of loading buffer and boiled for 2 minutes and immediately placed on ice. The RNA was separated by electrophoresis for 1 to 2 hours at 80 V on a 1% agarose gel containing 5% (v/v) formaldehyde. The gel was then soaked in 1XSSPE/50 mM NaOH for 15 minutes followed by two, 10 minute washes in 4X SSPE. The gel was then capillary blotted onto a Hybond-N nylon membrane for 12 to 20 hours and crosslinked at 80°C for 2 hours. Blots to be probed with anti-sense riboprobes were pre-hybridized for 1 to 12 hours at 60°C. Probe RNA was added directly to the pre-hybridization solution and incubated for 12 to 16 hours at 60°C. Membranes were then washed three times at room temperature for 30 minutes each in 2XSSPE/2%SDS and once at 65°C for 10 minutes in 0.2XSSPE/2%SDS. Blots to be probed with DNA probes were incubated at 42°C in DNA-pre-hybridization solution and then hybridized at 42°C for 12 to 16 hours. Blots were then washed in 1XSSPE/0.1%SDS for 15 minutes at room temperature followed by two more washes at 52°C.

βGal/DAPI Staining of Nematodes

Staining of nematodes for detection of β-galactosidase (βGal) activity was done essentially as described in Fire (1992). Worms were washed off seeded plates with M9 buffer and after pelleting, resuspended in 25 to 100 µl of M9 buffer. These were then pipetted onto poly-L-lysine coated 75 X 25 mm microscope slides and covered with 40 X 22 mm glass cover slips. The slides were coated by spreading 0.1 mg/ml poly-L-lysine onto the slides and incubating them for 30 minutes at 37°C. The slides were frozen on metal containers which were partially submerged in liquid Nitrogen for 15 minutes. The cover slips were then cracked off and the slides immediately submerged for 5 minutes in 100% acetone previously cooled to -20°C. The slides were washed for one minute each in 75%, 50% and 25% acetone and then allowed to dry briefly on the benchtop at room temperature. 100 µl of staining solution containing DAPI (see Appendix) was pipetted onto the slides and after 5 minutes, cover slips were added. The cover slips were sealed with nail polish and incubated at room temperature on moistened Whatman chromatography paper (3MM) in petri dishes for 2 to 24 hours. Animals were examined for βGal staining using brightfield microscopy and for DAPI staining using fluorescence microscopy.

RESULTS

Northern Analysis

The number, size and abundance of *fem-2* transcripts in the various stages and between males and hermaphrodites were estimated using Northern analysis. Northern blots containing approximately equal amounts of total RNA were probed with *fem-2* specific riboprobes. Levels of total RNA were estimated by comparing the loading of the lanes on the ethidium bromide stained agarose gels. *fem-2* mRNA levels were found to be the highest in the later four stages, L2, L3, L4 and adult. Lower levels of *fem-2* were also detected in eggs and L1 RNA (Figure 5). In order to determine if there were higher levels of *fem-2* mRNA in XO animals, RNA was compared between populations consisting entirely of XX animals versus those containing approximately 37% XO animals (due to a high incidence of males [*him-8*] mutation). No significant changes were observed in *fem-2* mRNA levels in the population containing XO adult or egg stages (Figure 5).

The blot shown in Figure 6 was produced by running an agarose gel for 10 hours at 50 V in order to separate any additional *fem-2* transcripts that are similar in size. No transcripts other than the 1.8 kb transcript predicted from cDNA analysis (Pilgrim *et al.*, 1995) were detected under these conditions. To test for a rare transcript, 15 µg of mixed stage (XX) poly(A) mRNA was separated by agarose gel electrophoresis, blotted and probed as described above (data not shown). This experiment also did not reveal any additional transcripts.

lacZ expression studies

A second approach to determine the expression patterns of *fem-2* was done by using a *lacZ* expression vector (Fire *et al.*, 1990). This is achieved by attaching the upstream regulatory sequences of *fem-2* to the *E. coli lacZ* gene (Figures 7 and 8). Following transformation of nematodes, production of a fusion protein that is functional for βGal activity will produce a staining pattern that mimics the expression pattern of *fem-2*. It must be noted that this may not reflect the normal expression pattern of FEM-2, it only represents where the transcript is present and the mRNA can be translated. Any effects due to introns or 3' nontranscribed sequences will be lost in this type of study. In addition, these vectors have not been successful detecting germ-line expression (Andy Fire, personal communication). The vector used for this fusion contains a multi-cloning site upstream of an expression cassette (Figure 7). This cassette contains the SV40 T antigen Nuclear Localization Signal (NLS), a small synthetic intron, 3 kbp of a *trpS::lacZ* fusion region (Hall *et al.*, 1982) and a 3' end cassette containing the C-terminal portion of the *unc-54* gene (uncoordinated, Fire *et al.*, 1990). The NLS can target a normally cytoplasmic protein such as βGal to the nucleus when placed at either end of the protein or at a variety of internal sites (Kalderon *et al.*, 1984). This results in more concentrated staining patterns but may not necessarily reflect the normal cellular localization of FEM-2 (Hirsh *et al.*, 1985). The synthetic intron is used because it has been observed that expression from some constructs in transgenic animals is more efficient from spliced than from unspliced transcripts (Buchman and Berg, 1988). The 3' portion of the *unc-54* gene is used because higher levels of expression were observed with *C. elegans* 3' end cassettes and the 3′ end of this gene does not play a role in its regulation (Fire *et al.*, 1990).

Five different constructs were engineered and injected in order to examine *fem-2* expression (Table 3, Figure 8). pDP#TJ01 contains 2 kbp of upstream sequences, the 5'UTR and the N-terminal 300 bp of *fem-2* ligated in frame to *lacZ*. pDP#TJ03 is identical to pDP#TJ01 except for a 4 bp insertion which causes a frameshift, resulting in a premature stop during translation of the *lacZ* portion of the gene. This will produce a fusion protein that lacks βGal activity and therefore represents a negative control. pDP#TJ04 and pDP#TJ05 are upstream deletions of pDP#TJ01 with pDP#TJ04 containing only 239 bp 5' to the beginning of the cDNA and pDP#TJ05 containing only 135 bp 5' to the beginning of the cDNA. A genomic clone (pDP#DBP141) containing the same 239 bp of upstream sequence as pDP#TJ04 has been shown to completely rescue all aspects of the strongest *fem-2* mutant (Pilgrim *et al.*, 1995). pDP#TJ04 was therefore constructed to observe what type of expression patterns it produced. pDP#DBP141 also contains 8.5 kbp of 3' genomic sequence which may be important in its phenotypic rescue. pDP#TJ07 was therefore produced by replacing the *unc-54* 3' end cassette in pDP#TJ04 with 1.5 kbp of *fem-2* sequence, including the C-terminal 17 amino acids, the 3'UTR and 988 bp of downstream sequence (summarized in Figure 8). Comparison of the staining patterns produced by this construct and pDP#TJ01 should reveal any role that the 3' end of *fem-2* plays in regulation.

Animals were transformed by co-injecting the fusion construct with a marker plasmid, pRF4 containing the mutant collagen gene, *rol-6(su1006)* which confers a dominant rolling behavior (Kramer *et al.*, 1990), into the syncytial gonads of adult hermaphrodites (Kimble *et al.*, 1982). A small fraction of the progeny (<0.2%) will take up the DNA and through recombination, create an extra-chromosomal array consisting of several

hundred copies of the injected plasmids (Mello *et al.*, 1991). F1 progeny that displayed the dominant marker phenotype were assumed to be carrying transgenic arrays comprised of approximately equal amounts of each plasmid. Five animals for each construct were selected and examined for the presence of the *lacZ* expression construct using PCR (Figure 9). F1 animals that transmitted the array (followed with the *rol* phenotype) to a portion of their progeny were then selected and maintained. For some of the constructs, DNA from the extra-chromosomal array was integrated into the genome in order to eliminate the mosaic animals from the analysis (Fire, 1986). The array was integrated by subjecting animals carrying the transgene to gamma irradiation, and screening for broods in which the array was inherited by all progeny. 300 rolling F2 progeny (irradiated DP91 animals are the P1 generation) were examined in this manner and two integrants were identified, DP101 and DP102 (identified at a frequency of 0.7 integrants/100 transgenic animals). The integrated line DP99 was isolated in a slightly different manner. DP103 adults were irradiated and maintained as any other strain. Two months later, a *rol-6* hermaphrodite was fortuitously observed to yield 100% rolling progeny and later verified to be an integrant (see Tables 2 and 3 for strains).

Transformation and βGal Staining of Nematodes

The results of the transformation experiments are summarized in Table 4. Transmitting lines were developed for all five constructs but resulted in mosaic animals. In addition, pDP#TJ01(DP101 and DP102) and pDP#TJ04 (DP99) were integrated into the genome. Animals from each of the 7 transgenic lines (5 carrying extra-chromosomal arrays and two with integrated sequences) were fixed and stained for βGal activity. DP90

(pDP#TJ01) showed the strongest staining of the strains carrying extra-chromosomal arrays (Figures 10-12). Many of the animals displayed mosaic staining patterns presumably due to the instability of the array. This problem was overcome by either combining the results of a large number of stained animals or by using the integrated strain DP98. Once a portion of an array is integrated into the genome it is inherited in a Mendelian pattern which results in much more stable staining patterns than extra-chromosomal arrays. Staining was observed in four major times and areas in strains DP90 or DP98. Staining was first identified prior to the comma stage of embryo development (300-400 minutes, fertilization=0). This is shortly before sexual dimorphism can be first identified in the developing embryo (470 minutes, Sulston *et al.*, 1983). One cell begins to stain shortly before a set of 16-20 cells (Figure 10, A and B). The identity of the single cell is not known but a possible candidate is the excretory cell, which is involved in osmoregulation (White, 1988) The position of the 16-20 cells staining in the embryo is consistent with that of the intestinal precursor cells. This pattern of staining continued until approximately the L1/L2 molt (Figures 10 and 11). The next stages, L2 to adult show reduced staining in the presumed intestinal cells with the reduction in staining beginning with the central intestinal cells and spreading to the outside cells until eventually the adult showed staining in the anterior and posterior intestinal cells only. The resolution is insufficient to directly determine if the early embryonic staining is restricted to one sex or another. Two lines of evidence suggest that staining occurred in both XO and XX animals. Populations consisting of only XX animals showed embryonic staining which shows that the staining is not restricted to XO animals only. Homozygous integrated lines containing approximately equal numbers of XO and XX animals showed

almost 100% of the animals staining, demonstrating that both are expressing *fem-2/lacZ* in the putative intestinal cells.

Staining was also observed in the head and tail regions of XX and X0 animals beginning in L2 and continuing into adulthood (Figures 11 and 12). The anterior staining was variable and sometimes included cells of the pharynx. Tail staining was also variable and was clustered around the anus with some staining extending into the tail spike of XX animals. Finally, staining was observed in the distal tip cells (DTC) of adult males only.

DP103 (containing pDP#TJ04) also showed staining patterns that were similar to those observed with DP90. This staining was much fainter (usually not appearing until at least 24 hours of staining while DP90 staining would appear after 1 hr) and a higher percentage of the worms did not stain at all. DP105 (pDP#TJ05), DP106 (pDP#TJ03) and DP107 (pDP#TJ07) did not show any staining at any stage (see Tables 1 and 2 for the extra-chromosomal arrays carried in each strain). Animals carrying these constructs were left in the staining solution for up to 48 hours a number of times and no staining was ever observed.

DP91 was used to generate two integrated lines (DP101 and DP102) by gamma irradiation. These were isolated in the same screen but from different plates and therefore assumed to be independent. DP102 was backcrossed with wild-type males to remove the *fem-1; him-8* background (producing strain DP98) and examined for βGal activity. It demonstrated similar staining patterns to DP90 but with much less variability. DP106 was also used to generate an integrated line (DP99). This strain did not show any staining at all, as was expected from the DP106 results.

Analysis of Sex Determination Mutants carrying *edEx26*

The reporter transgene was placed in background of other sex determination mutations to examine whether these mutants could affect the staining patterns and feminization of the germline phenotype. Only *fem-1(hc17)*, *fem-3(q20gf)* and *tra-2(b202)* altered the staining patterns of *fem-2*:βGal (Table 5). *fem-1(hc17)* and *fem-3(q20gf)* mutant backgrounds caused staining in the distal tip cells (DTCs) of the gonad in XX animals as well as XO animals (Figures 15 and 16). At both the restrictive and permissive temperatures. Conversely, *tra-2(b202)* XX masculinized animals did not show DTC staining (Figure 17). XX animals normally do not show DTC staining, but this result reveals that *tra-2* can masculinize XX animals without producing XO-like DTC staining. All other staining was unaffected. *tra-2(e1940mx)*, *fog-2(q71)* and *fem-2(b245)* transgenic animals did change the staining patterns at any temperature. *fem-2(q117)* was not examined for staining patterns.

Feminization of the Germline

Examination of DP90 also revealed that transgenics showed a dominant 'feminization of the germline' (FOG) phenotype. XX animals carrying *edEx26* were self-sterile but were capable of out-crossing with males (Table 4), showing that oocyte production is unaffected. XO animals were somatically male with a normal gonad and tail but had oocyte-like cells present in their germlines (Figures 13 and 14). The putative oocyte nuclei stained intensively with DAPI, suggesting a polyploid DNA content (Figure 14, C). Endoreduplication of DNA is characteristic of oocyte production in a male gonad (T. Schedl, personal communication). This FOG phenotype was not observed with the other transgenic strains carrying extra-chromosomal

arrays. The integrated line of *edEx26* (DP98) also showed the dominant FOG effects. The brood sizes of XX animals from other transgenic strains were found not to differ significantly from the wild-type brood sizes (Table 4), suggesting that sperm production was not affected, as sperm production is limiting for production of self-progeny (Wood, 1988).

Suppression of the FOG Phenotype

Each of the mutants mentioned above were also tested for their ability to suppress the FOG phenotype of transgenic animals carrying *edEx26*. *tra-2(b202)*, *fem-1(hc17)*, *fem-3(q20gf)* and *fem-2(b245)* alleles were all capable of suppressing the FOG phenotype, even when heterozygous (Table 5). This semi-dominant suppression transformed the females into self-fertile hermaphrodites at 15°C. Homozygous strains of *fem-1(hc17)*, *fem-3(q20gf)* and *fem-2(b245)* also suppressed the FOG phenotype at 15°C (a *tra-2(b202) edEx26* strain was not isolated, Table 5). At 25°C *fem-1(hc17)* and *fem-2(b245)* worms carrying *edEx26* showed complete feminization of XX animals and incomplete feminization of XO animals. XO animals had a feminized germline and gonad but had only partially feminized tails (seemingly identical to XO tail of *fem-1(hc17)* single mutants). These mutants carrying the transgene did not show a more extreme feminization than the single mutants. *tra-2(b202); edEx26* worms at 25°C were partially masculinized pseudomales producing only sperm in their germline and partially masculinized tails. This phenotype appears to be identical to that of non-transgenic *tra-2(b202)* mutants and demonstrates that the *tra-2(b202)* phenotype is epistatic to the *edEx26* FOG phenotype. A small percentage of *fem-3(q20gf) ; edEx26* worms raised at 25°C were self-fertile, producing small brood sizes. This suggests that the extra *fem-3* activity caused by *fem-*

3(q20gf) mutants can help compensate for the *edEx26* FOG phenotype. *fem-2(q117)*, *tra-2(e1940mx)* and *fog-2(q71)* were unable to suppress the FOG phenotype at any temperature when either heterozygous or homozygous (*fem-2(q117)* was not examined when homozygous).

DISCUSSION

There is little known about how *fem-2* acts to direct spermatogenesis and male somatic development. Especially perplexing is how zygotic and maternal product interact and how this product is regulated in XO and XX animals. This study approached these questions by determining when and where *fem-2* mRNA is present and by attempting to correlate these observations with the previously determined temperature sensitive periods (Kimble *et al.*, 1984).

Northern analysis of the number and size of *fem-2* transcripts

Northern blots probed with *fem-2* did not reveal any additional sex specific or stage specific transcripts to the 1.8 kb transcript (Figures 5 and 6), consistent with the previous evidence of a single transcript (Pilgrim *et al.*, 1995). This is similar to the situation seen with *fem-1* where only one transcript has been identified on Northern blots and in the screening of two cDNA libraries (Spence *et al.*, 1990).

Despite the above evidence, it is formally possible that another 1.8 kb transcript exists. For example, analysis of the expression of *fem-3* has revealed three similarly sized transcripts of 1.7, 1.62 and 1.55 kb, possibly varying in length of the poly(A) tail (Ahringer *et al.*, 1992). *tra-2* transcripts include a major transcript of 4.7 kb, and three other transcripts of similar size, 1.80, 1.85 and 1.90 kb (Okkema and Kimble, 1991). The 1.85 and 1.80 kb transcripts are identical except for the length of their poly(A) tails (Okkema and Kimble, 1991). RNase protection studies have also revealed *her-1* uses multiple capping sites, and several poly(A) addition sites. This results several transcripts differing in size by a few bases (Perry *et al.*, 1993). It is

possible that a rare, similarly sized *fem-2* transcript may have been overlooked. The cDNA library that revealed only one *fem-2* transcript was constructed in a fashion that clones only a small portion of the poly(A) tail and therefore would not have identified any additional poly-adenylation (Barstead and Waterston, 1989).

It is possible that a second transcript is present in very low quantities for a very short period of time and was therefore overlooked on the Northerns and the cDNA library screen. The 1.55 kb transcript from the *fem-3* gene, while observable on Northerns, is only found in L4 and adult animals and has not been found in the cDNA library (Rosenquist and Kimble, 1988, Ahringer *et al.*, 1992). Zarkower and Hodgkin (1992) were able to detect two *tra-1* transcripts using Northern analysis but only one was identified in cDNA screens. The functionally important *her-1* transcript (Trent *et al.*, 1991) has also not been detected in cDNA libraries but has been detected on Northern blots (Perry *et al.*, 1993). Therefore, if a second *fem-2* transcript was rare enough to be undetectable on Northerns, it most likely would not have been present in the cDNA library either. This would be especially true if the second transcript happened to be XO specific as the cDNA library was constructed from XX animals (Barstead and Waterston, 1989).

These examples aside, it seems improbable that there is more than one transcript produced from the *fem-2* locus. If a second transcript does exist, its nature is such that it coincidentally escaped detection by three different techniques. Ten genes involved in sex determination have had their mRNA levels analyzed and five have revealed only one transcript (*sdc-1*, Nonet and Meyer, 1991; *sdc-2*, Nusbaum, Brenner and Meyer, personal communication; *sdc-3*, Klein and Meyer, 1993; *fem-1*, Spence *et al.*,

1990 and *fem-2*). Of the remaining five, three have revealed more than one transcript but one transcript is necessary and sufficient for wild-type function (*her-1*, Perry *et al.*, 1993; *xol-1*, Rhind *et al.*, 1995; *tra-1*, Zarkower and Hodgkin, 1992). Analysis of *tra-2* and *fem-3* have also not revealed any role for the additional transcripts (Okkema and Kimble, 1991; Rosenquist and Kimble, 1988). The simplest explanation for our data is that one transcript is sufficient and necessary for the sex, stage and tissue specific roles of *fem-2* (Pilgrim *et al.*, 1995; this work).

Developmental Expression of *fem-2*

Northern blots revealed that *fem-2* mRNA is highly expressed from the second larval stage to adults in XX animals. Very low levels are also seen in egg and L1 stages indicating that there is at least some *fem-2* mRNA present in all stages of XX development. This is similar to the results found for a number of sex determination genes. *fem-1* and *sdc-2* mRNA levels are relatively constant throughout development (Spence *et al.*, 1990, Nusbaum *et al.*, personal communication). *fem-3* RNA is easily detectable in embryo, L4 and adult hermaphrodites but is also present at low levels in L1, L2 and L3 animals (Rosenquist and Kimble, 1988). *sdc-1* mRNA levels are highest in embryo and L1 but also detectable in all other stages (Nonet and Meyer, 1991). *tra-2* expression shows that the 4.7 kb transcript and either the 1.9 or 1.8 kb transcript are found at all stages of development (Okkema and Kimble, 1991). Therefore, *fem-2* like many other sex determination genes is actively transcribed in all developmental stages.

The peak levels of *fem-2* mRNA correspond well with its position in the sex determination pathway. "Upstream" genes such as *xol-1*, *sdc-1*, *sdc-3* and *her-1* (see Figure 3) show highest mRNA levels early in development

(embryos, L1 and L2, Rhind *et al.*, 1995; Nonet and Meyer, 1991; Klein and Meyer, 1993; Perry *et al.*, 1994). The high levels of *fem-2* expression in L2 and L3 may represent the time in the XX germline when *tra-2* is modulated, thereby allowing *fem-2* expression in order to direct the brief period of spermatogenesis. Kimble *et al.* (1984) has shown that the TSP for *fem-2* mutants for the XX germline is between late L1 and L2 which is consistent with the beginning of the increased levels of *fem-2* mRNA. The high levels found in L4 and adults might represent the production of mRNA or protein for inclusion in oocytes. A number of sex determination genes show a maternal effect and of the six that have had their mRNA levels analyzed (*sdc-1,2,3* and *fem-1,2,3*), all but *sdc-3* show significant mRNA levels in adults (Klein and Meyer, 1993). The maternal packaging of the yolk proteins by the intestine has been observed to take place in L4 and adult animals (Kimble and Sharrock, 1983) and packaging of the products of the sex determination genes may take place at a similar time. This is also consistent with the formation of the first oocytes at the L4/adult molt (Kimble and Ward, 1988). The low levels in egg and L1 may represent the activity responsible for controlling the early somatic decisions (consistent with the TSP of the gonadal soma, Kimble *et al.*, 1984). It is difficult to determine how much of the variation in levels can be attributed to differences in loading and this should be analysed in the future using *in situ* antibody staining.

I have not been able to detect any observable increase in mRNA levels on Northern blots comparing populations containing 37% XO animals when compared to XX populations (Figures 5, 6 and unpublished results). This is similar to the results observed for *fem-3* and *fem-1* (Rosenquist and Kimble, 1988; Spence *et al.*, 1990). A possible explanation

for this is that *fem-2* is equally expressed in XX and XO, and differences between the sexes depend upon post-transcriptional control of *fem-2* itself or its target.

In summary, evidence suggests that a single transcript of 1.8 kb is produced in XX animals from the second larval stage onward. No differences were observed in transcript levels between populations containing 37% XO animals and those consisting of almost entirely XX animals.

βGal Staining of Transgenics

The second approach used to examine *fem-2* expression levels was to examine the staining patterns produced by a *fem-2/lacZ* fusion construct, allowing for temporal and spatial analysis of expression. Transgenic animals carrying the 5′ end of *fem-2* and 2 kbp of upstream sequences attached to the *lacZ* expression vector have shown staining in four areas at specific times. These include the head and tail regions in L2 and older animals, the intestinal precursor cells of early developmental stages and the distal tip cells of the adult XO gonad (Figures 10, 11 and 12). Staining was localized to specific cells whenever possible by using DAPI staining of nuclei followed by observation of fluorescence. The identity of the cell was then estimated using the published embryonic and post-embryonic cell lineages and positions of cells (Sulston and Horvitz, 1977, Sulston *et al.*, 1983).

Intestinal Precursor Cell Staining

Intestinal precursor cell staining was the strongest in embryos, L1 and L2 animals of both sexes (Figures 10 and 11). The role of the intestine in sex determination is unclear. It is involved in packaging the yolk proteins into

the developing oocytes in L4 and adult hermaphrodites (Kimble and Sharrock, 1983). Perhaps the intestinal cells synthesize *fem-2* mRNA early in development and store it for packaging into the developing oocytes. The intestine has also been observed to interact with the germline precursor cells (Z2 and Z3). Electron micrograph reconstructions of the 470 minute embryo show that the germ cells are connected cytoplasmically and protrude large lobes into intestinal cells INT5L and INT5R (Sulston *et al.*, 1983). This association ends at approximately the time of hatching. It is possible that the high levels of *fem-2* mRNA/protein in the intestine at this time are packaged directly into the developing germ cells. One problem with this model is that there is no need for all of the intestinal cells to express *fem-2* unless they are connected to each other in some way. There is no reason why the intestinal staining would extend into L1 and L2 because by this time the connection with the germ cells has been broken. Finally, this would not explain staining in both XX and XO animals. Northern blot analysis shows the highest levels of *fem-2* transcript in the later stages of development and this is assumed to be accumulation of product for maternal packaging. This suggests that these high levels do not represent the staining observed in the intestinal cells.

Some transgenic *lacZ* expression constructs have been shown to produce ectopic intestinal and/or pharyngeal staining when insufficient upstream sequences are present (A. Fire and I. Hope, personal communication). It is therefore possible that the intestinal staining is artefactual and the *fem-2* product for maternal packaging is produced in the germline. βGal staining in the germline of transgenic animals has never been observed (A. Fire, personal communication). Perhaps the mRNA for

fem-2/lacZ is present in the germline but cannot be translated, or is translated but inactive.

Distal Tip Cell Staining

Staining was also observed in the distal tip cells (DTC) of adult XO gonads (Figure 12). The staining corresponds to the extended TSP observed in XO animals for the maintenance of sperm production (Kimble *et al.*, 1984). This *fem-2* product may be responsible for prolonging spermatogenesis in XO animals. DTCs play an important role in germ cell development. Laser ablation studies have revealed that they are necessary in both XO and XX animals for preventing the germ cells at the distal end of the gonads from entering meiosis prematurely (Kimble and White, 1981), resulting in a reduced number of germ cells, most of which are sperm. Recently it has been demonstrated that mitosis is maintained by a ligand encoded by the *lag-2* gene binding with the germline specific membrane receptor, GLP-1 (Henderson *et al.*, 1994). Mutations in either *lag-2* or *glp-1* result in a similar phenotype to laser ablation of the DTCs (Kimble and Ward, 1988). The DTCs are also responsible for guiding the migration of the arms of the gonads in XX animals, a role performed by the linker cell in XO animals (Kimble and Ward, 1988). How FEM-2 produced in the DTCs might maintain spermatogenesis in the XO germline is not known. The predicted protein sequence of FEM-2 does not suggest that it is a secreted protein (Pilgrim *et al.*, 1995) which would imply that DTC expression of FEM-2 may trigger the secretion of another protein that promotes spermatogenesis (Figure 18). Alternatively, its role may be to prevent the expression of another DTC protein that acts to promote oogenesis. For example, the *mog* genes are thought to negatively regulate *fem-3* by binding to its 3' UTR

(Graham and Kimble, 1993). In XO animals, *fem-2* may act to prevent the release of one of the *mog* products from the DTCs. *fem-2;mog-1* double mutants have been observed to produce sperm suggesting that *mog* mutations can suppress *fem-2* mutations (Graham and Kimble, 1993). *fem-3* gain of function mutants have been observed to be capable of suppressing the *fem-2(b245)* phenotype which is consistent with this model (Barton *et al.*, 1987). The somatic gonad is a good candidate for controlling the sexual fate of the germ cells but until now, little evidence was present to support its role. It was known that the sheath cells of the hermaphrodite gonad were involved in controlling the arrest of the oocytes, as oocytes produced in a male gonad (which lacks sheath cells) undergo endoreduplication (T. Schedl, personal communication). The βGal staining observed in this work is the first evidence that in addition to the other roles of the somatic gonad, it may also be involved in determining the sex of the germ cells via the DTC.

Additional Staining

The remainder of the βGal staining that is observed may represent sexually dimorphic cells. The chemical fixing of animals necessary for XGal staining disrupts the cell boundaries, reducing the resolution of DIC optics and makes it difficult to identify individual cells in an animal. The earliest of this "unidentified" staining takes place at approximately the same time that the intestinal cell staining first appears (Figures 10 and 11). Based upon its position in the embryo, this staining could represent either the cephalic companion cells of the male or an excretory cell involved in osmoregulation (Sulston *et al.*, 1983).

There is also considerable staining in the head and tail regions of L2 and older animals (Figures 11 and 12). There are no sexually dimorphic cells identified in the head region outside the nerve ring (Sulston and Horvitz, 1977). The male nervous system has not been completely reconstructed and it is assumed that many of the 294 neurons common to both sexes may have different circuitry in males and hermaphrodites. The staining observed in the head may therefore represent some of these neurons. Much of the head staining is also found in the pharynx which does not display any sexual dimorphism at all (Sulston and Horvitz, 1977). I am assuming that this is ectopic staining caused by the nature of the transgene (A. Fire, personal communication).

The staining observed in the tail regions is consistent with the position of a number of sexually dimorphic cells (Figures 11 and 12). These cells include the ectodermal cells B, Y, U and F which are all involved in the postembryonic development of the male tail (Sulston and Horvitz, 1977, Sulston *et al.*, 1983). The sexually dimorphic cell T is also found in the tail and is involved in the development of the lateral hypodermis (Sulston and Horvitz, 1977, Sulston *et al.*, 1983). These cells begin their post-embryonic cell divisions between L1 and L3 (Sulston and Horvitz, 1977) and it is possible that *fem-2* activity in these cells is necessary to promote male development.

The observation that *fem-2* is also expressed in the head and tail regions of hermaphrodites, even though it functions in male somatic development could be explained if it is transcriptionally active in a large number of sexually dimorphic cells. The presence of FEM-2 in these cells would not necessarily cause male development, but instead give those cells

the option of developing as male or female, depending on whether the appropriate target gene product, such as FEM-3 is present or active.

Regions of *fem*-2 Necessary for Staining

In order to determine which 5' and/or 3' regions are necessary for proper expression, five constructs were tested for their ability to produce βGal staining in transgenic animals (Table 4 and Figure 8). The strongest staining was observed in worms carrying *edEx26* (DP90). This array contained pDP#TJ01 which has 2 kbp of upstream sequence. Transgenic animals carrying *edEx27* (pDP#TJ04 in strain DP103) with only 239 bp of *fem-2* upstream sequence showed weaker and more variable staining than *edEx26*. The staining observed with *edEx27* was in the same cells as with *edEx26*. It is possible that the 1.8 kbp of extra upstream region in TJ01 contains an element that increases the efficiency but not the tissue specificity of the *fem-2* promoter. A genomic clone containing the entire *fem-2* gene attached to the same 239 bp of upstream sequence (pDP#DBP141) is capable of completely rescuing all phenotypes of *fem-2* null alleles *(b245e2005)* when present in an extra-chromosomal array (Pilgrim *et al.*, 1995). The small amounts of *fem-2* product produced by pDP#DBP141 may be as efficient for rescue as the higher levels produced by a larger upstream construct, but the same levels of *fem-2/lacZ* transcripts may not be detectable. Another possible explanation is that *edEx27* contains only a few copies of pDP#TJ04. Transgenic animals for all arrays were examined for the presence of constructs using PCR (Figure 9) but it is impossible to quantify the number of copies from the data I have. It is possible to quantify the number of constructs in an extra-chromosomal array or integrated into the genome using genomic Southern analysis (Mello *et al.*, 1991). However, only low

levels of correlation have been observed between copy number and expression levels in extra-chromosomal arrays and integrated sequences have been observed to be susceptible to position effects (MacMorris *et al.*, 1994). This makes it difficult to make any assumptions regarding copy number and expression levels.

Three other constructs did not show any staining at all. *edEx30* (pDP#TJ07) is identical to *edEx27* (pDP#TJ04) except the 3' end of the *unc-54* gene has been replaced with that of *fem-2*. This plasmid was constructed to investigate the possibility that the *fem-2* 3' UTR plays a role in post-transcriptional regulation as has been demonstrated with *tra-2* and *fem-3* (Goodwin *et al.*, 1993, Ahringer and Kimble, 1991). It is difficult to draw any conclusions from this result due to the poor staining seen with *edEx27*. Because of this, the lack of staining seen with *edEx30* may be due to the presence of the 3' end of *fem-2* or it may be because there are fewer copies of it in *edEx30* then there are of pDP#TJ04 in *edEx27*. This experiment should be repeated in the future using the complete upstream region that was used in *edEx26* animals.

pDP#TJ05 has only 136 bp of upstream sequence. This construct did not produce any staining at all and may not contain enough upstream sequence to do so. As expected, pDP#TJ03 did not show any staining. It was constructed with the *lacZ* gene out of frame, in order to serve as a negative control.

In addition, integration of either pDP#TJ01 or pDP#TJ04 into the genome did not drastically alter the staining patterns other than the observation that DP98 eliminated the mosaicism observed with *edEx26*. The integrated lines did not show a reduction in the levels of staining, despite the likelihood that integrated strains contain fewer copies than those

carrying extra-chromosomal arrays. This is perhaps because only a few copies are necessary for visible staining.

Staining Patterns in Sex Determination Mutants

edEx26 (pDP#TJ01) in *fog-2(q71)*, *tra-2(e1940mx);vab-9(e1744)*, and *fem-2(b245)* mutant backgrounds did not show any differences in staining patterns (Table 5). *tra-2(b202)* staining was also the same as wildtype but due to its masculinized phenotype, suggests that *tra-2(b202)* can masculinize an XX animal without affecting *fem-2* expression in the distal tip cells (Figure 17). This casts doubt on any previous hypothesis that *fem-2* activity in the distal tip cells is responsible for maintaining spermatogenesis in males. Further evidence against this is the observation that an XO adult with the DTC ablated produce sperm (Kimble and Ward, 1988). Regarding the other mutations that also did not affect staining, the obvious conclusion is that these mutations do not affect the temporal or spatial patterns of *fem-2* mRNA or protein product localization. This experiment is only capable of identifying mutations that affect *fem-2* localization if they interact with the *fem-2* upstream region, 5' UTR or the part of the gene encoding the N-terminal 100 amino acids. It is possible that some of these mutations do regulate *fem-2* localization but do so through regions of *fem-2* that are not present in the *fem-2/lacZ* fusion construct.

edEx26 in *fem-3(q20)*; *dpy-20(e1282)* and *fem-1(hc17)*; *him-8(e1489)* mutant backgrounds resulted in βGal staining in the distal tip cells of adult XX animals at permissive and restrictive temperatures (Table 5) while all other staining patterns were unchanged (Figures 15 and 16). The FEM-2/βGal DTC staining observed in XO animals suggests that *fem-2* activity in these cells plays a role in preventing the switch to oogenesis that is observed

in XX animals. The observation that a *fem-3* gain of function mutation causes additional βGal staining in the distal tip cells of XX animals suggests that *fem-3* may be involved in the regulation of *fem-2* (Figure 19).

The observation that a *fem-1* loss of function mutant causes FEM-2/βGal expression in the distal tip cells of XX animals is more difficult to rationalize. One possibility is that FEM-2 and FEM-1act as a complex that is negatively regulated through FEM-1. In wildtype XX animals, FEM-2 could be inactivated in the DTCs by negative regulation of the complex through FEM-1. In *fem-1(hc17ts)* mutants, the repressor may not be able to bind to FEM-1 properly and therefore FEM-2 may remain active. Another possibility is that FEM-1 and wildtype FEM-2 interact to promote spermatogenesis normally and this same interaction between FEM-1 and FEM-2/βGal may prevent the fusion protein from staining. This might be avoided by *fem-1(hc17)* mutants, which may not be able to bind FEM-2 properly, thereby allowing FEM-2/βGal to stain. If this were true, then the staining may not be indicative of when FEM-2 is active, but instead of when it is not bound to FEM-1.

In summary, βGal staining was observed at four significant times and places in transgenic animals. This includes the intestinal precursor cells and cells in the head and tail regions of XX and XO animals. Staining was also observed in the DTC of adult XO animals. Evidence in the literature using mRNA *in situ* hybridizations strongly supports that this type of staining represents when the endogenous mRNA is transcribed (Henderson *et al.*, 1995). It can therefore be assumed that this experiment provides a good picture of where and when *fem-2* is transcribed.

Feminization of the Germline (FOG)

During the analysis of strain DP90 it was observed that animals carrying the *edEx26* array displayed strong feminization of the germline. Most XX animals were self-sterile (produced no sperm) and some XO animals showed oocyte-like cells in their germlines. There were no observable signs of somatic feminization. This is a significant finding as it represents the first evidence that the germline and somatic roles of *fem-2* are separately mutable. Does *fem-2* function differently in the germline or soma or does it act the same way but cause a different result? For example, it may act on TRA-1 in the soma and some other factor in the germline. Alternatively, it may act on FEM-1 in both tissues but FEM-1 may have different substrates in each. The finding of the FOG phenotype represents the first step towards separating the germline and somatic functions of *fem-2* and may help to identify domains involved in each. This could be done by examining *fem-2/lacZ* constructs that contain different portions of *fem-2* for their ability to feminize the germline and soma of transgenic animals.

This dominant negative phenotype was also observed in strain DP98 which had pDP#TJ01 integrated into the genome. A number of other constructs (pDP#TJ03, -04, -05 and -07) were tested and were not observed to cause the same feminization, either by examination of the animals or by brood size analysis (Table 4).

The cause of the germline feminization is not known. It is possible that a large excess of upstream sequence titrates away germline specific factors necessary for wildtype *fem-2* expression. In order to test this hypothesis, animals containing pDP#TJ03 were examined. The only difference between this construct and pDP#TJ01 is that the attached *lacZ* gene is out of frame with the *fem-2* protein. Transgenic animals carrying

this construct were not feminized, suggesting that translation of the fusion is necessary for this phenotype, not the mere presence of upstream sequences. Alternatively, it may be that without the large portion of the *lacZ* gene, the short protein is unstable and therefore unable to produce the dominant negative effect. pDP#TJ01 produces a fusion protein with βGal which might be able to poison the complex that *fem-2* interacts with. pDP#TJ03 produces a small fusion protein because the out of frame *lacZ* gene results in a nonsense codon shortly downstream. A number of other constructs have also been observed to cause the same phenotype (P. Jäckle-Baldwin and D. Pilgrim, personal communication). pDP#DBP023ΔBam is a genomic clone spanning *fem-2* that has had the four central bases of the *Bam*HI site removed. This deletion takes place at the splice site between exons 3 and 4 in the *fem-2* gene (Pilgrim *et al.*, 1995). This construct only feminizes in *fem-2(b245)* or *fem-2(q117)* backgrounds. Presumably this affects splicing and either causes a frameshift or removes a portion of the protein. This construct also causes the FOG phenotype. A similar phenotype has been observed with *fem-1*, *fem-3* and *tra-2* (J. Kimble, personal communication), suggesting a general rather than a specific mechanism.

Interactions with Sex Determination Mutants

While examining the effects of the different sex determination mutations of staining patterns, the effects on the feminization of the germline were also analyzed. The results can be grouped into three classes, the phenotype when heterozygous for the sex determination mutant, the phenotype when homozygous at the permissive temperature and the phenotype when homozygous at the restrictive temperature. Seven

different sex determination mutants were tested in at least one of these categories (Table 5).

fog-2(q71), *fem-2(q117)* and *tra-2(e1940mx)* did not suppress the FOG phenotype under any circumstances. At the permissive temperature, four of the mutations, *fem-2(b245ts)*, *tra-2(b202ts)*, *fem-3(q20sd,gf,ts)* and *fem-1(hc17ts)* were able to at least partially suppress the FOG phenotype when heterozygous. These mutations resulted in XX animals capable of self-fertilization and XO animals that did not show any oocytes in their germlines. When these mutants were examined as homozygotes, *fem-2(b245)*, *fem-1(hc17)* and *fem-3(q20)* also showed the same suppression at the permissive temperature. *tra-2(b202)* homozygous animals carrying *edEx26* at the permissive temperature were not isolated.

The mechanism by which these four mutants suppressed the FOG phenotype is not known. Somehow they were able to prevent the mutant FEM-2/βGal product from interfering with wildtype FEM-2. For masculinizing mutants such as *tra-2(b202)* and *fem-3(q20gf)* it is reasonable to assume that even at permissive temperature they were able to help increase the wildtype *fem-2* levels. *tra-2(b202)* mutants at permissive temperature may be partial loss of function mutants which allow for higher levels of wildtype *fem-2*. One of the roles of *tra-2* is negative regulation of the *fems* (See Figure 4) and partial loss of this function would increase the levels of *fem-2*. This may allow the wild-type FEM-2 to better compete with FEM-2/βGal and suppress the FOG phenotype. *fem-3* gain of function mutants may act in much the same way.

fem-1(hc17) and *fem-2(b245)* may act in a different manner. Both of these are temperature sensitive mutations which suggests that there are conformational changes to the proteins. This may affect the binding of

these proteins and this could be the cause of the suppression if they interact directly with *fem-2* or something that regulates it. One possible model for *fem-1* is that mutant FEM-1 protein preferentially binds to wildtype FEM-2 rather than FEM-2/βGal resulting in a higher number of active complexes. Similar possibilities exist for *fem-2(b245)*. Wild-type FEM-2 may compete better than the mutant FEM-2 for binding with targets. A question that arises is that if *fem-1(hc17)* and *fem-2(b245)* proteins had increased binding affinity, why are they temperature sensitive loss of function mutants? It is possible that this better binding is a disadvantage under normal circumstances but when in competition with mutant FEM-2/βGal can be advantageous.

tra-2(e1940,mx) and *fog-2(q71)* are not temperature sensitive mutations which may explain why they were unable to suppress the FOG phenotype. This does not in any way suggest that neither interacts directly with FEM-2 or its regulators, only that their respective mutations do not affect their function in such a way to alter FEM-2 enough to over-ride the FOG phenotype.

Four of the five temperature sensitive mutants were also examined at the restrictive temperature for their effects on the FOG phenotype. *fem-2(b245)* and *fem-1(hc17)* animals carrying *edEx26* showed identical phenotypes to each of the single mutants. Each were able to suppress at the permissive temperature but not at the restrictive temperature, as expected. Even if their mutations free FEM-2 from the dominant effects of FEM-2/βGal they retain so little activity that they cannot rescue the phenotype. Neither *fem-1(hc17)* or *fem-2(b245)* completely feminize XO animals at the restrictive temperature and one possibility was that when carrying *edEx26* they might show complete feminization. This is seen in *fem-1(hc17); fem-*

2(b245) double mutants which are completely feminized at the restrictive temperature even though neither by themselves can do so (Kimble *et al.*, 1984). No enhancement of the feminization phenotype however was observed.

Analysis of *tra-2(b202)* mutants carrying *edEx26* at the restrictive temperature showed complete masculinization of XX animals. This suggests that by removing *tra-2* activity, which negatively regulates the *fem* genes, not only does it allow the wildtype *fem-2* product to overcome the dominant negative effects of *edEx26*, but the wildtype *fem-2* is also able to continually promote spermatogenesis. It is possible that loss of *tra-2* function greatly increases the levels of wildtype FEM-2 such that it can now compete with the high levels of FEM-2/βGal mutant protein.

The last mutant analysed, *fem-3(q20gf)* was able to partially suppress the effects of *edEx26* at the restrictive temperature. Animals raised at 25°C were sometimes able to produce small amounts of self-progeny. This is also observed with *fem-3(q20gf); fem-2(b245)* double mutants (Barton *et al.*, 1987) suggesting that each mutation is able to suppress the other enough to result in some spermatogenesis and some oogenesis. It appears that *edEx26* is able to combat the increased activity of FEM-2 caused by *fem-3(q20gf)* enough to produce a small number of oocytes. This is the opposite of the permissive temperature when *fem-3(q20gf)* is able to partially block the effects of *edEx26* in order to produce a few sperm.

The work described in this paper raises questions and proposes hypotheses that require further experiments to test. The most important experiment to be done is mRNA *in situ* hybridizations to further analyse the *fem-2* levels. This experiment will help to support the expression vector results and also determine if the germline is producing any *fem-2*

mRNA. Further analysis on mRNA levels in XO animals is also required along with examination of *fem-2* mRNA levels in a number of mutant backgrounds. The discovery of the FOG phenotype also demonstrates the need for experiments to study the parts of *fem-2* necessary to cause this phenotype and to understand the nature of it.

CONCLUSION

fem-2 mRNA levels were examined using Northern Blot analysis. Only one transcript of approximately 1.8 kb in size was observed. Low levels were seen in egg and early larval stages and elevated levels were observed in the later four stages, L2, L3, L4 and adults. There were no higher levels observed in populations containing 37 % XO animals. This suggests that a single transcript of *fem-2* is capable of controlling both somatic and germline sex determination. The abundant levels observed in the later stages are consistent with packaging of a maternal product and when spermatogenesis is observed. The low levels observed early in eggs and L1 coincide with the first signs of sexual dimorphism and may play a role in early somatic differentiation.

The expression of *fem-2* was also analyzed by attaching the 5' end of the *fem-2* gene (including putative regulatory regions) to the *lacZ* reporter construct. Staining in transgenic animals was first observed in XX and XO embryos shortly before the comma stage. This staining was localized to two areas, one consistent with the intestinal precursor cells and the other with unidentified cells. This staining was present until L2 when additional cells showed staining. These cells were located in the head and tail regions of L2 to adult animals of both sexes. Staining was also observed in the distal tip cells of adult males which is consistent with the extended temperature sensitive period observed for *fem-2* for the maintenance of sperm production (Kimble *et al.*, 1984). The distal tip cells are known to be involved in controlling the mitosis/meiosis switch of germ cells in the distal portion of the gonad (Kimble and Ward, 1988). The observation of staining in the distal tip cells suggests that through *fem-2* they may also be

involved in determining whether cells switch from mitosis to spermatogenesis or oogenesis. The observation that *fem-1* and *fem-3(gf)* mutants can alter the staining patterns in the distal tip cells further supports this theory.

The *fem-2* reporter construct caused feminization of the germline of transgenic animals. Analysis of several sex determination mutants revealed that some were able to suppress this phenotype. The information obtained in this study has helped to understand the mechanisms behind *fem-2* activity. It is now clearer when *fem-2* activity may be required and which cells it may be active in.

TABLES

Table 1. *C. elegans* strains used in this work.

Strain[a]	Genotype
CB1489	*him-8(e1489)*
CB3347	*fem-1(hc17ts); him-8(e1489)*
CB3366	*tra-2(b202ts)*
CB3535	*fem-2(b245ts)*
CB3538	*fem-2(b245ts); him-8(e1489)*
CB3807	*tra-2(e1940dm); vab-9(e1744)*
CB4002	*fem-3(q20 sd,ts); dpy-20(e1282ts)*
CB4108	*fog-2(q71)*
DP71	*fem-2(q117ts); him-8(e1489)*
DP90	*edEx26*(pDP#TJ01; pRF4)
DP91	*fem-1(hc17ts); him8(e1489); edEx26*(pDP#TJ01;pRF4)
DP92	*fem-3(q20sd, ts); dpy-20(e1282ts); edEx26*(pDP#TJ01;pRF4)
DP93	*tra-2(e1940dm); vab-9(e1744); edEx26*(pDP#TJ01;pRF4)
DP94	*tra-2(b202ts); edEx26*(pDP#TJ01;pRF4)
DP95	*fog-2(q71); edEx26*(pDP#TJ01;pRF4)
DP96	*fem-2(q117ts); him-8(e1489); edEx26*(pDP#TJ01;pRF4)
DP97	*fem-2(b245ts); him-8(e1489); edEx26*(pDP#TJ01;pRF4)
DP98	*edIs3*(pDP#TJ01;pRF4)
DP99	*edIs4*(pDP#TJ04;pRF4)
DP101	*fem-1(hc17ts); him8(e1489); edIs2*(pDP#TJ01;pRF4)
DP102	*fem-1(hc17ts); him8(e1489); edIs3*(pDP#TJ01;pRF4)
DP103	*edEx27*(pDP#TJ04;pRF4)
DP105	*edEx28*(pDP#TJ05;pRF4)
DP106	*edEx29*(pDP#TJ03;pRF4)

DP107	*edEx30*(pDP#TJ07;pRF4)
JK663	*fem-2(q117ts)*

Source

[a] CB strains constructed in the MRC lab in Cambridge, U.K.

DP strains constructed in this laboratory.

JK strains constructed in the laboratory of J. Kimble, University of Wisconsin

Table 2. Plasmid vectors used in this study.

Plasmid	origin and/or construction
pDP#AMc18	1.75 kbp *fem-2* cDNA in pBluescript
pDP#DBP026	6 kbp genomic fragment cloned into pBluescript
pDP#DBP063	1.5 kbp *Hind*III/*Sal*I genomic fragment cloned into pBluescript
pDP#DBP101	4.5 kbp *Sst*I/*Xho*I genomic fragment cloned into pBluescript
pDP#DBP141	10 kbp genomic fragment cloned into pBluescript
pDP#TJ01	2.5 kbp *Sal*I/*Xho*I genomic fragment containing the promoter and 5' end of *fem-2* from pDP#DBP101 cloned into pPD22.04 (*Sal*I site)
pDP#TJ02	2.5 kbp *Pst*I/*Sal*I fragment from pDP#TJ101 cloned into pBluescript
pDP#TJ03	2.5 kbp *Sal*I/*Xho*I fragment from pDP#DBP101 cloned into pPD22.11 (*Sal*I site)
pDP#TJ04	600 bp *Sal*I/*Pst*I fragment from DP#DBP141 cloned into pDP#TJ01 (replacing the 2.5 kbp *Sal*I/*Pst*I fragment)
pDP#TJ05	2 kbp *Hind*III fragment deleted from pDP#TJ01
pDP#TJ06	1.5 kbp *Apa*I/*Spe*I fragment from pDP#DBP063 containing the 3'UTR of *fem-2* and downstream sequences cloned into pPD16.01
pDP#TJ07	3.1 kbp *BssH*II/*Apa*I fragment from pDP#TJ06 cloned into pDP#TJ03 (replacing the 2.4 kbp *BssH*II/*Apa*I fragment)
pPD16.01	*lacZ* expression vector (Fire *et al.*, 1990)

pPD22.04	*lacZ* expression vector (Fire *et al.*, 1990)
pPD22.11	*lacZ* expression vector (Fire *et al.*, 1990)
pRF4	4 kbp *EcoR*I fragment of *C. elegans* genomic DNA containing the *rol-6 (su1006)* collagen gene cloned into pBluescribe (Kramer *et al.*, 1990)

Table 3. Summary of micro-injections.

plasmids injected	worms injected	survivors	F1 rollers	transmitting lines
pRF4	50	28	25	1
pDP#TJ03/pRF4	184	82	13	2
pDP#TJ01/pRF4	267	92	4	1*
pDP#TJ04/pRF4	180	105	150	24
pDP#TJ05/pRF4	27	23	29	1
pDP#TJ07/pRF4	23	19	6	1
totals	731	349	227	30

*transgenic animals carrying this array show strong βGal staining and are feminized (germline only).

Table 4. Summary of transgenic lines and their characteristics.

Constructs	Transgenic lines	Detectable βGal expression?	XX Brood size (n)**	oocytes present in XO gonad?
pDP#TJ01*	DP90	Yes	0(50)	Yes
integrant	DP101	Yes	0(25)	Yes
	DP102			
pDP#TJ04*	DP103	Yes***	252(12)	No
integrant	DP99	No	238(7)	No
pDP#TJ05*	DP105	No	275(12)	No
pDP#TJ03*	DP106	No	292(11)	No
pDP#TJ07*	DP107	No	N/A	No

*these constructs were carried as extra-chromosomal arrays in these transgenic lines

**wildtype hermaphrodites have a brood size of 274-374 self-progeny, 12 animals examined, mean of 329 (Hodgkin, 1983), n=number of animals examined, N/A=not assayed.

***this strain did exhibit βGal staining but it was faint and variable

Table 5. Effects of sex determination mutants on *edEx26* transgenics.

A) Effects on *fem-2* staining patterns by sex determination mutations.

No effect	Causes DTC staining in XX
fem-2 (b245ts)	*fem-1 (hc17ts)*
fem-2 (q117ts)	*fem-3 (q20ts,gf)*
fog-2 (q71)	
tra-2 (e1940mx)	
*tra-2 (b202ts)**	

**-tra-2(b202ts)* XX animals were incompletely masculinized but did not show distal tip cell staining

B) Sexual phenotypes

Mutant	single mutant	single mutant carrying *edEx26*	
background	phenotype 25°C[a]	20°C	25°C
fem-2 (b245ts)	feminized	some self-fertile	feminized
fem-2 (q117ts)	feminized	some self-fertile[b]	N/A[b]
fem-1 (hc17ts)	feminized	self-fertile	feminized
fem-3 (q20gf,ts)	mog	most self-fertile	few fertile
tra-2 (b202ts)	masculinized	some self-fertile[c]	masc[c]
tra-2 (e1940mx)	mixed (mostly fog)	fog	fog
fog-2 (q71)	fog	fog	fog

[a]-feminized=XO/XX females, mog=masculized germline, fog=feminized germline

[b]-*fem-2(q117ts)* was not examined as a homozygote

[c]-*tra-2(b202ts)* homozygotes were only isolated at 25°C as masculinized XX animals, the phenotype at 20°C is when heterozygous

FIGURES

Figure 1: Diagrams of adult *C. elegans*. A, -wildtype hermaphrodite; B, -wildtype male; C, *-fem-1* female; D, -XX pseudomale showing a feminization of the germline (FOG) phenotype (modified from Hodgkin, 1988; White, 1988).

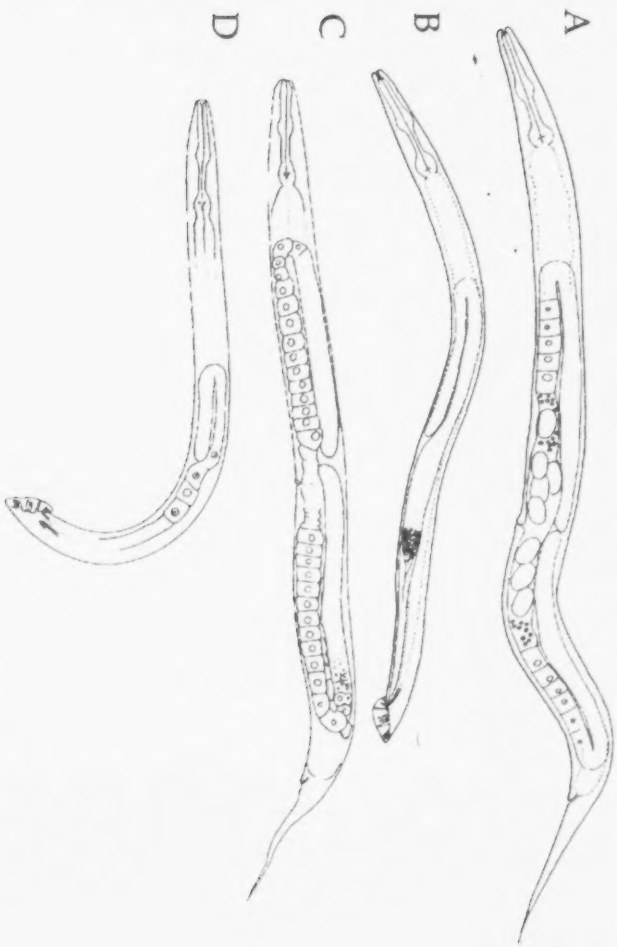

A
B
C
D

Figure 2: Gonadogenesis and the adult anatomy in hermaphrodites and males. A, -development of the gonad and germline in hemaphrodites (left) and males (right). Somatic tissue is black and germline tissue is clear. Empty circles=early meiotic nuclei; Black circles=primary spermatocyte nuclei; Empty squares=oocyte nuclei; Stippled squares=sperm; Mitotic regions of germline tissue are blank. L1 to L4=larval stages, eL4=early L4, lL4=late L4, A=adult; B, -position of the Distal Tip Cells (DTC) with respect to the germ cells, Mit=mitotic cells, P=cells in pachytene, G=gamete-forming cells, S=somatic cells (adapted from Kimble and White, 1981).

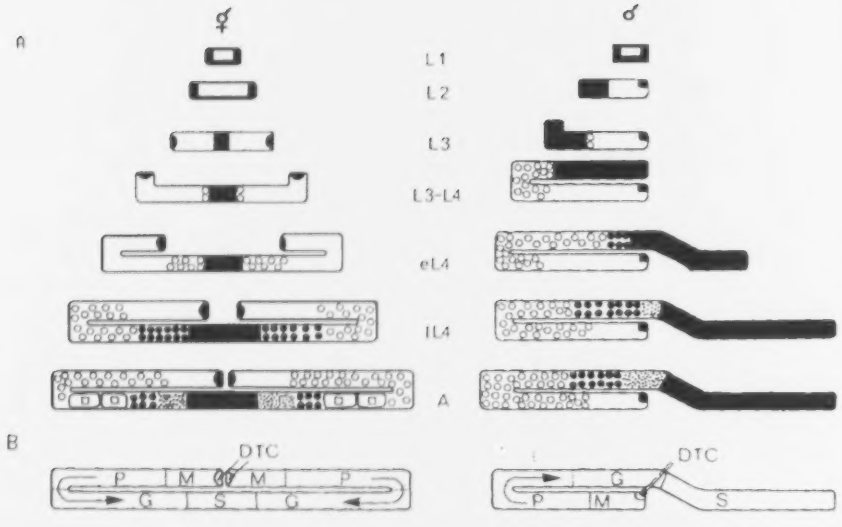

A
♀
♂
L1
L2
L3
L3-L4
eL4
lL4
A
B
DTC
P
M
M
P
G
S
G
DTC
G
P
M
S

Figure 3: Model of genetic interactions based on epistatic interactions between the genes involved in somatic and germline sex determination in *C. elegans*. Pointed arrows indicate positive regulation and barred arrows signify negative regulation. Genes contained within the shaded area are germline specific (modified from Hodgkin, 1992).

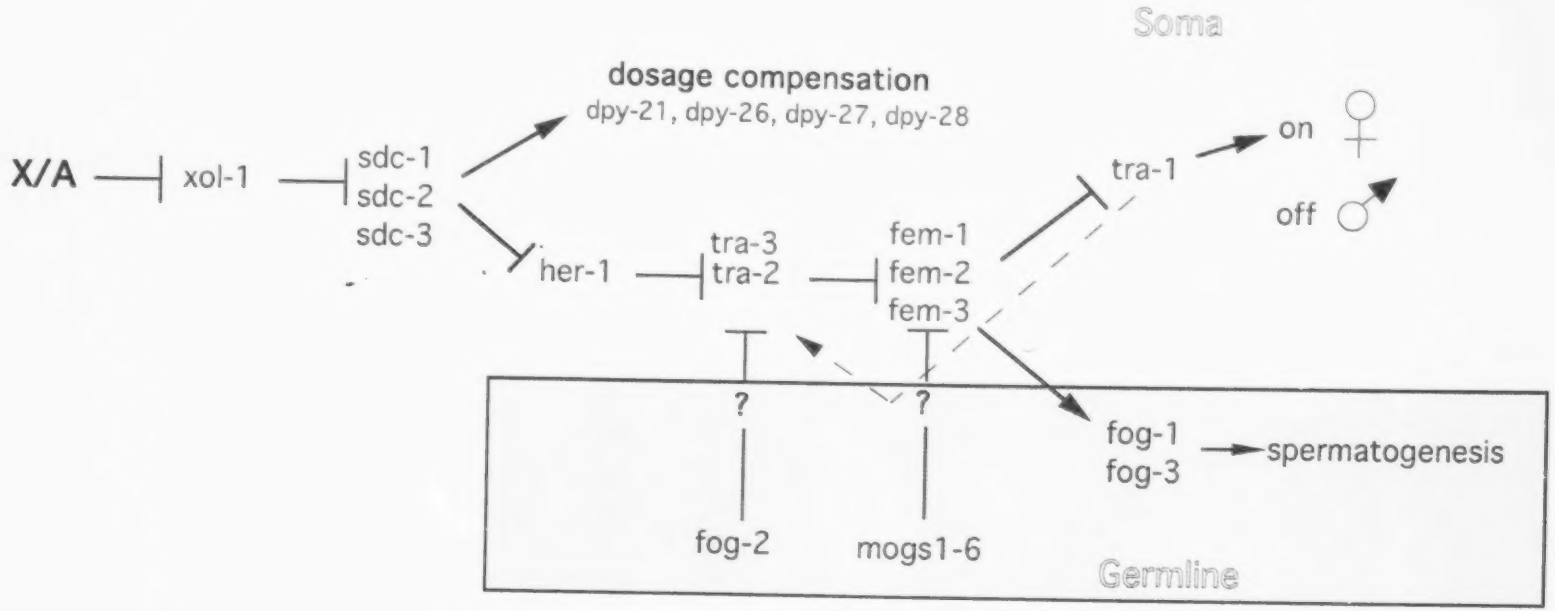

Soma
dosage compensation
dpy-21, dpy-26, dpy-27, dpy-28
X/A
xol-1
sdc-1
sdc-2
sdc-3
her-1
tra-3
tra-2
fem-1
fem-2
fem-3
tra-1
on
off
?
?
fog-1
fog-3
spermatogenesis
fog-2
mogs1-6
Germline

Figure 4: Diagram illustrating the molecular model of the signal transduction pathway involved in somatic sex determination (adapted from Kuwabara and Kimble, 1992). HER-1 is depicted as a diffusible ligand (Perry *et al.*, 1993), TRA-2 as a trans-membrane protein with an extracellular binding domain for HER-1 and an intracellular binding domain for FEM-3 (Kuwabara *et al.*, 1992), FEM-1 as an intracellular protein containing Ankyrin repeats shown here as being capable of binding to TRA-1 (Spence *et al.*, 1990), FEM-3 as an intracellular protein with a binding domain for TRA-2 (Ahringer *et al.*, 1992), FEM-2 as a phosphatase (Pilgrim *et al.*, 1995) and TRA-1 as a transcription factor containing zinc finger domains (Zarkower and Hodgkin, 1992), P=potential phosphorylation sites, barred arrows indicate negative regulation, pointed domains on TRA-2 and FEM-3 represent TRA-2/FEM-3 binding domains, rounded domains on TRA-1 and FEM-1 represents an interaction between FEM-1 and TRA-1, the scissors on FEM-2 represent its phosphatase activity. TRA-3 has been ommitted from this diagram.

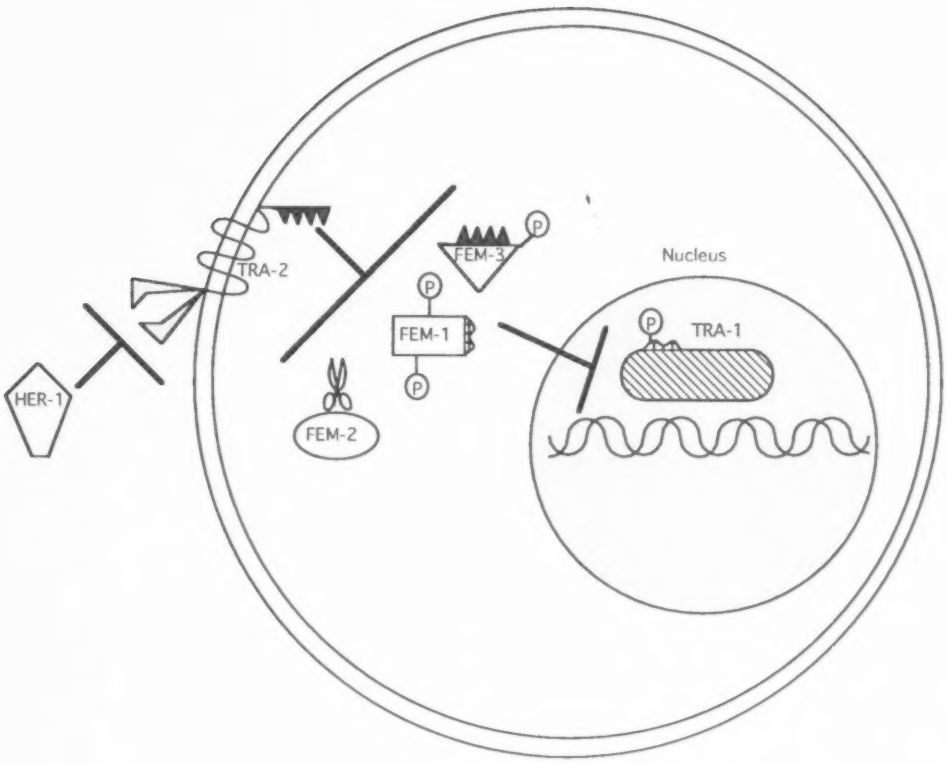

TRA-2
FEM-3
P
Nucleus
P
FEM-1
P
TRA-1
P
HER-1
FEM-2

Figure 5: RNA blot analysis. 15 ug of total RNA isolated from developmentally staged N2 (99.8% XX) or *him-8(e1489)* populations (37% XO) was separated on 1% agarose gels for 2 hours at 80 Volts, transferred to Hybond N membranes and probed with an antisense *fem-2* riboprobe. Panel A, -lanes A-F, egg, L1, L2, L3, L4 and adult XX RNA respectively. Panel B, -lane G, XX egg RNA, Lane H, *him-8(e1489)* egg RNA, Lane I, XX adult RNA. Photographs of the ethidium bromide stained agarose gel are presented to represent the amount of RNA in each lane (bottom panels). The blot in panel B was probed under more sensitive conditions (the probe had a higher specific activity) than the blot in panel A, accounting for the differences in band intensity.

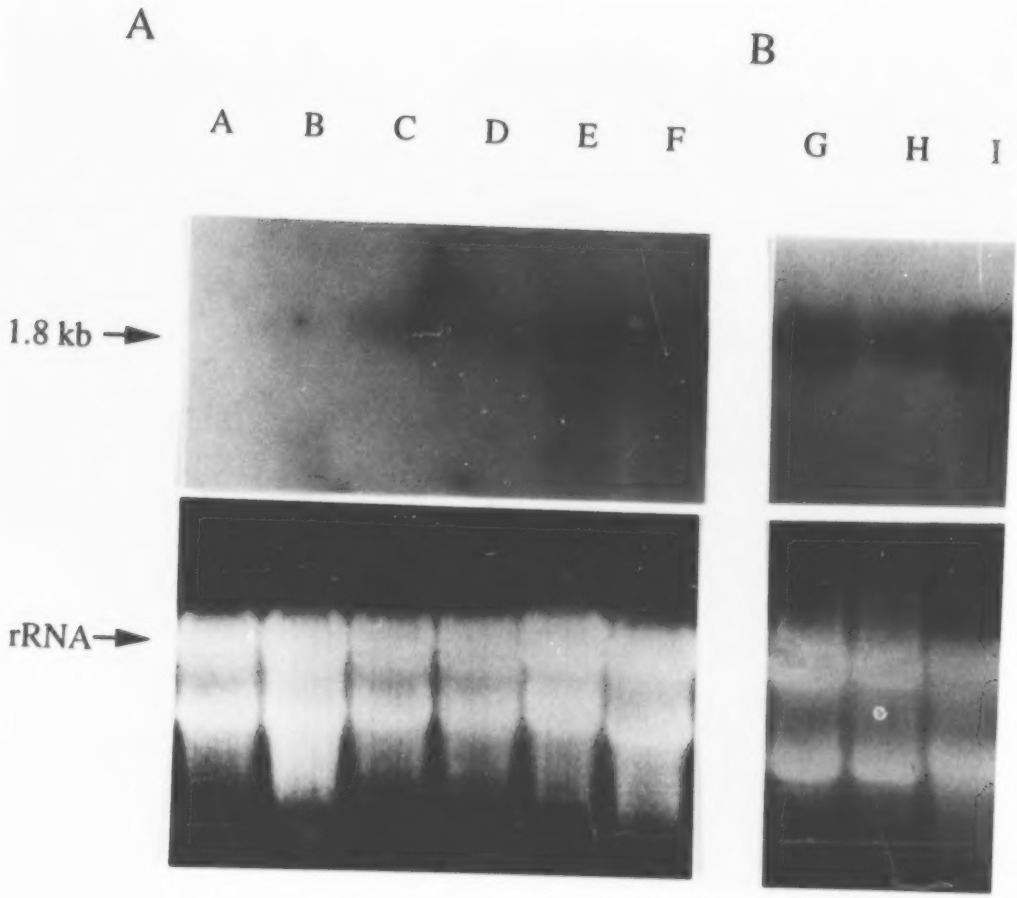

A
B
A B C D E F G H I
1.8 kb
rRNA

Figure 6: Northern blot of a 1% agarose gel containing formaldehyde electrophoresed for 10 hours at 50 V for maximum separation. Lanes A-G, 15 ug of egg, L1, L2, L3, L4, adult and mixed stage total RNA from XX animals. Lanes H-J, 15 ug of mixed stage, adult and egg total RNA from a *him-8(e1489)* strain containing approximately 37% XO animals. The lanes were not loaded equally as this gel was not intended to compare levels of *fem-2* mRNA between developmental stages. Some lanes were over or under loaded in order to search for additional *fem-2* transcripts. Lanes E and I were significantly contaminated with bacterial RNA (determined by the observation of two sets of rRNA bands on the ethidium stained gel, data not shown).

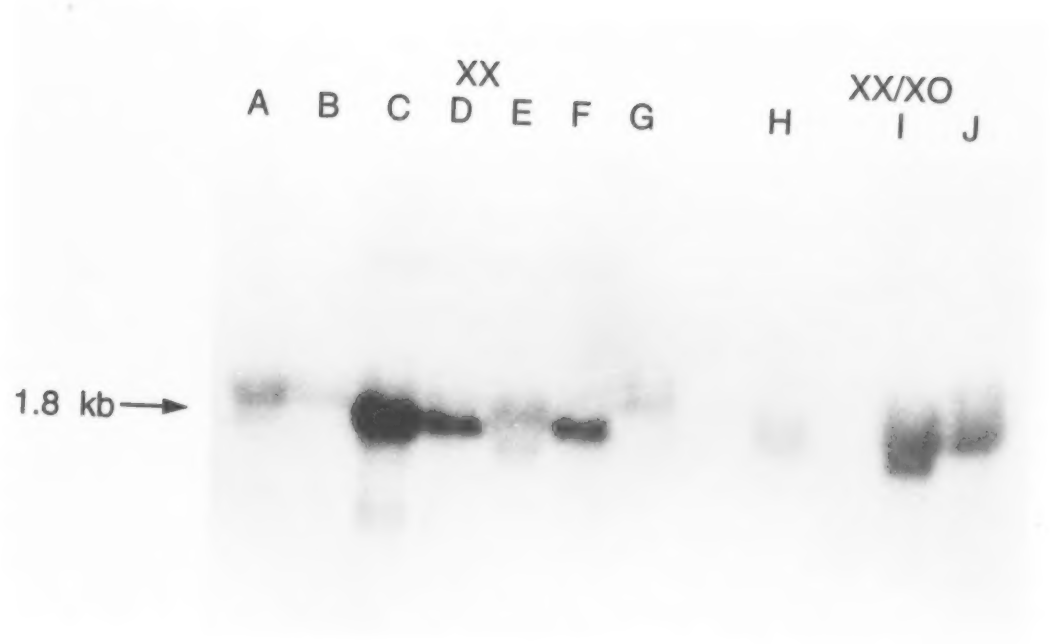

XX
XX/XO
A
B
C
D
E
F
G
H
I
J
1.8 kb

Figure 7: A, -partial restriction map of genomic DNA containing *fem*-2, B, -the structure of the *fem*-2 gene indicating the intron/exon boundaries and the location of the six motifs conserved with other protein phosphatases (Pilgrim *et al.*, 1995), C, -the structure of the *lacZ* expression vector (Fire *et al.*, 1990). The vertical dashed line indicates the portion of *fem*-2 used for the expression studies. The letters I, II, III, IV, V and VI indicate the position of the six motifs that *fem*-2 shares with other known PP2C protein phosphatases. MCS-multi cloning site, NLS-nuclear localization signal,

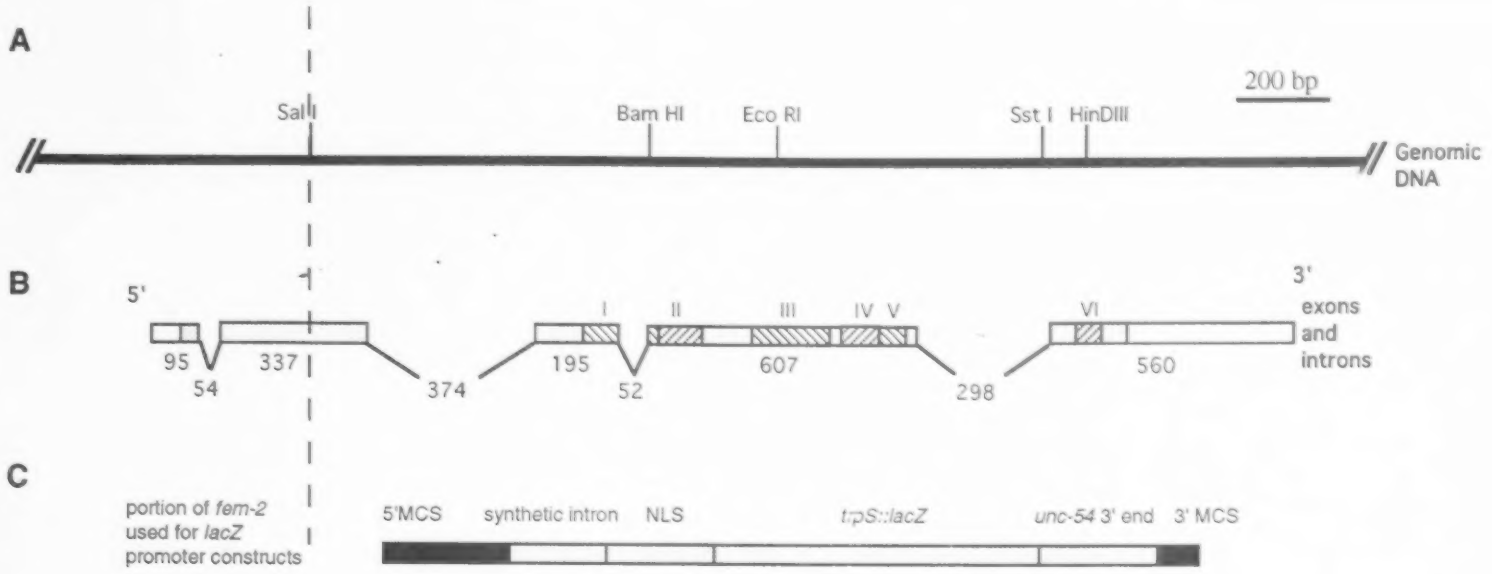
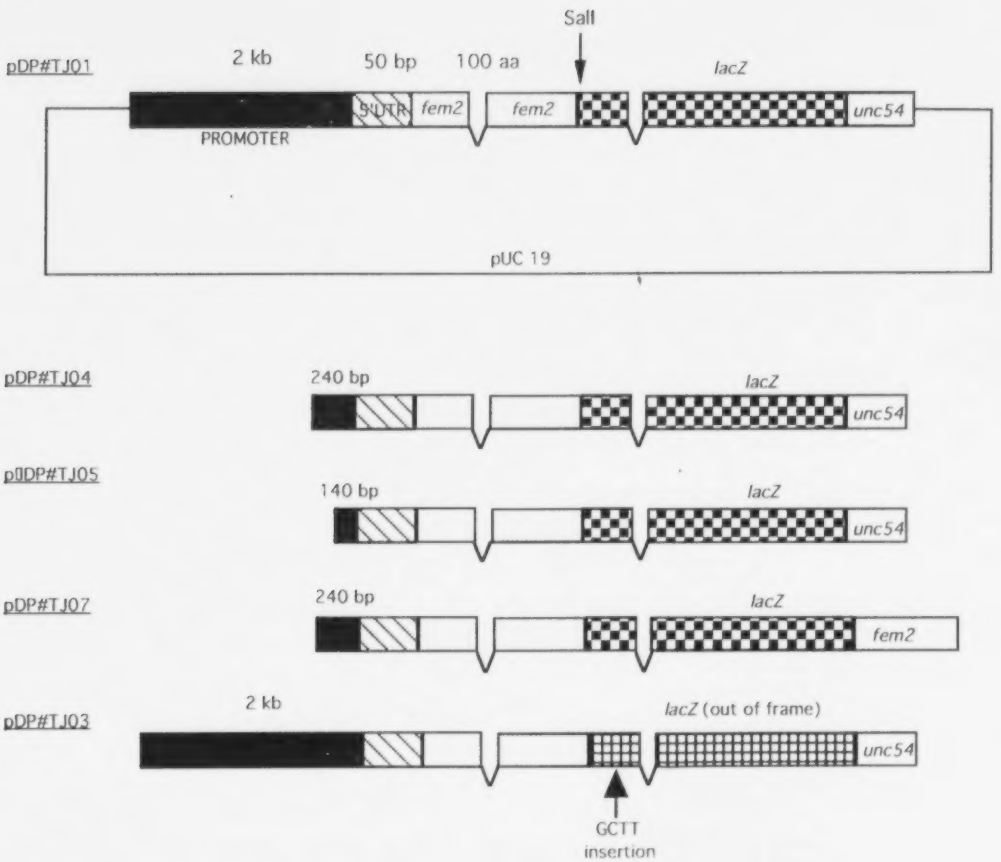

A
200 bp
Sal I
Bam HI
Eco RI
Sst I
HinDIII
Genomic DNA
B
5'
1
95
54
337
374
I
II
III
IV V
195
52
607
298
VI
560
3'
exons and introns
C
portion of fem-2 used for lacZ promoter constructs
5'MCS
synthetic intron
NLS
tpS::lacZ
unc-54 3' end
3' MCS

Figure 8: Diagram of the five expression constructs used to analyse *fem-2* expression. A, -structure of the five constructs showing the differences in the promoter sequence, the *lacZ* sequence and the 3' ends. B, -comparison of the *unc-54* and *fem-2* 3' end cassettes.

A


SalI
pDP#TJ01
2 kb
50 bp
100 aa
lacZ
5'UTR
fem2
fem2
unc54
PROMOTER
pUC 19
pDP#TJ04
240 bp
lacZ
unc54
pDP#TJ05
140 bp
lacZ
unc54
pDP#TJ07
240 bp
lacZ
fem2
pDP#TJ03
2 kb
lacZ (out of frame)
unc54
GCTT
insertion

B

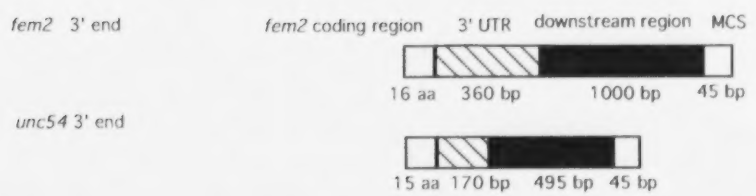

fem2 3' end
fem2 coding region
3' UTR
downstream region
MCS
16 aa
360 bp
1000 bp
45 bp
unc54 3' end
15 aa
170 bp
495 bp
45 bp

Figure 9: Photograph of an agarose gel demonstrating PCR of small numbers of transgenic animals. The numbers on the side indicate the sizes in kbp of the lambda size markers in lane A. The remaining four lanes indicate the products of the polymerase chain reaction using reverse primer which is located in the 5'MCS of pPD22.04 and pPD22.11, and TGJO1 which is located in the 5' untranslated region of the *fem-2* gene. Template DNA used was B, -purified genomic DNA from five DP106 nematodes, C, -genomic DNA from wildtype animals not carrying an extra-chromosomal array, D, -genomic DNA from five DP90 animals, E, -purified pDP#TJ01. The DNA band observed at 2.2 kbp in lanes B, D and E corresponds to the distance between the two primers in pDP#TJ01 and pDP#TJ03.

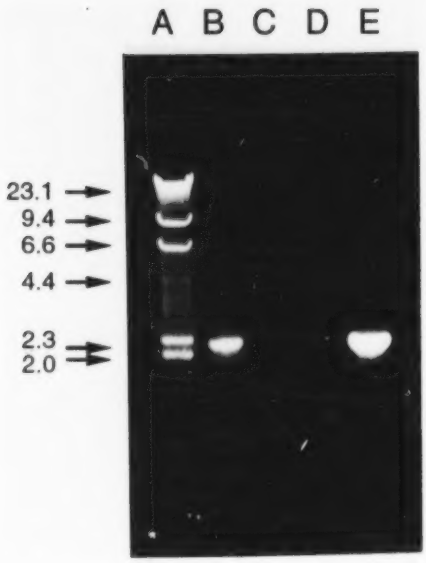

A B C D E
23.1
9.4
6.6
4.4
2.3
2.0

Figure 10: Photomicrographs of βGal and DAPI staining of DP98 (*edIs2*, pDP#TJ01). Animals were co-stained with XGal and DAPI. A, -an embryo at approximately the 200-300 cell stage showing βGal staining in one cell; B, -DAPI staining of the same embryo in A showing the number of nuclei and the approximate position of the staining seen in A using fluorescence microscopy; C, -an embryo at approximately the comma stage of development showing βGal staining in a number of cells whose position is consistent with the intestinal precursor cells; D, -an embryo at the three-fold stage of development showing βGal staining in the intestinal precursor cells; E, -a just-hatched L1 animal showing the same βGal staining as described above.

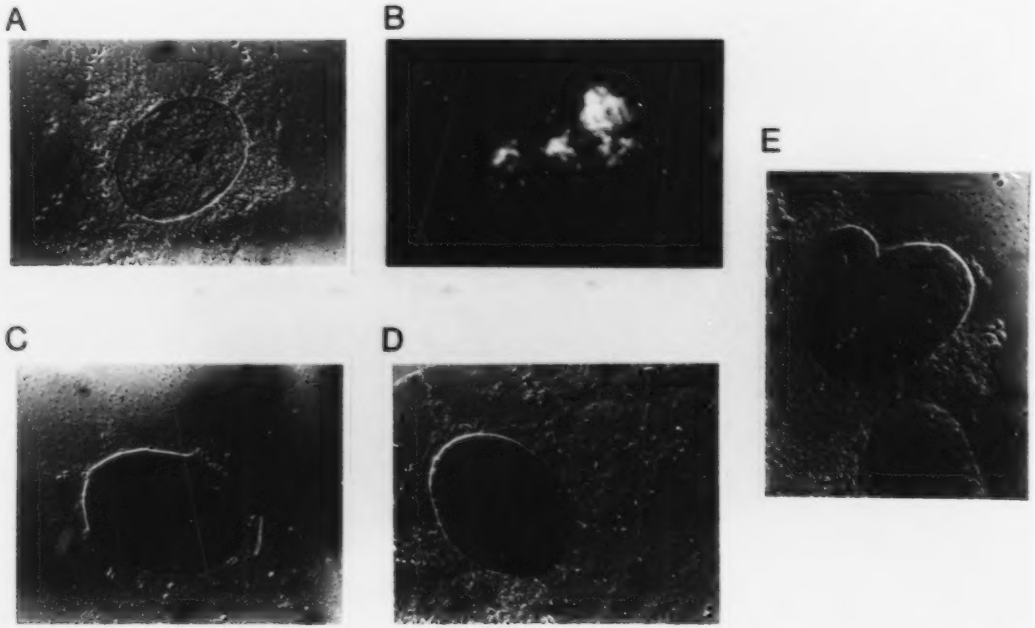
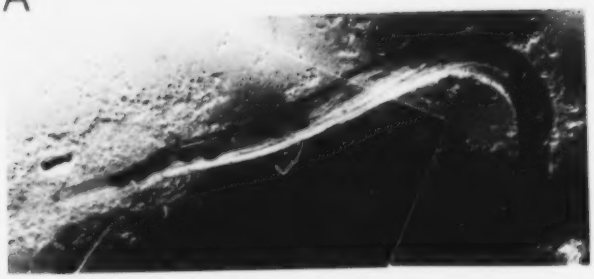
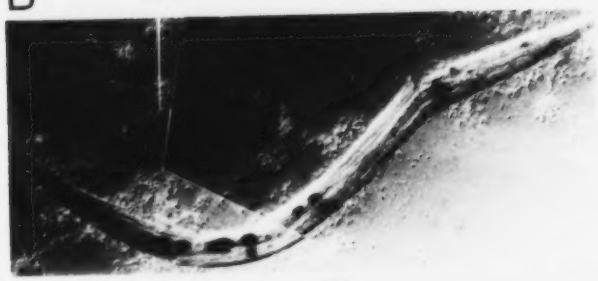

A
B
C
D
E

Figure 11: Photomicrographs of βGal and DAPI staining of DP98 (*edIs2*, pDP#TJ01) larval stage and adult XX animals. Animals were co-stained with XGal and DAPI. A and B, -composite photomicrographs of L2 animals using bright-field microscopy showing βGal staining of the intestinal cells as well as some cells in the head; C and E, -adult XX animals showing higher magnifications of the βGal head and tail staining respectively using bright-field microscopy; D and F,- DAPI staining of the same animals in C and E except the nuclei were visualized using fluorescence microscopy.

A

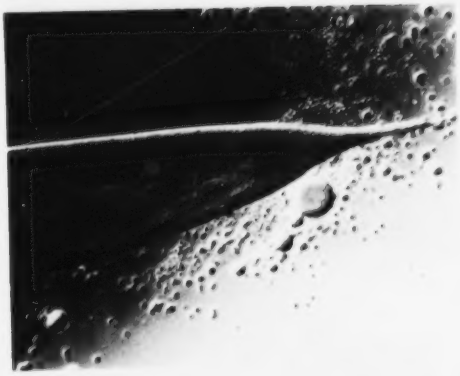


B

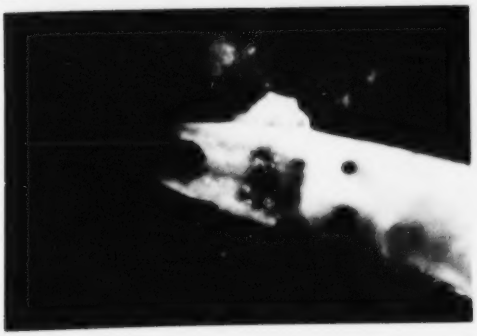


C

E



D



F

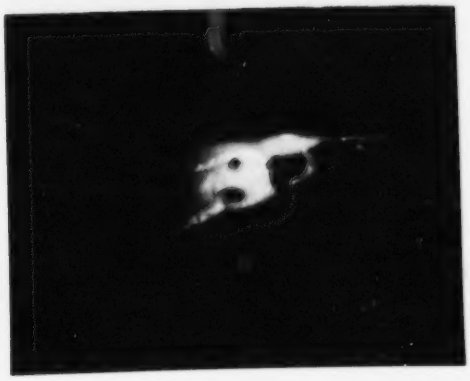


Figure 12: Photomicrographs of βGal and DAPI staining of DP98 (*edIs2*, pDP#TJ01) XO adults. A, -βGal staining of an adult XO tail; B, D and F, -βGal staining of the head, gonad and extruded gonad of XO animals; C, E and G -DAPI staining of the same animals as B, D and E showing positions of the nuclei. The arrows in D and F are pointing to the staining observed in the distal tip cells of the gonad. The gonad is extruded from the body in F and G to better visualize the location of the βGal staining.

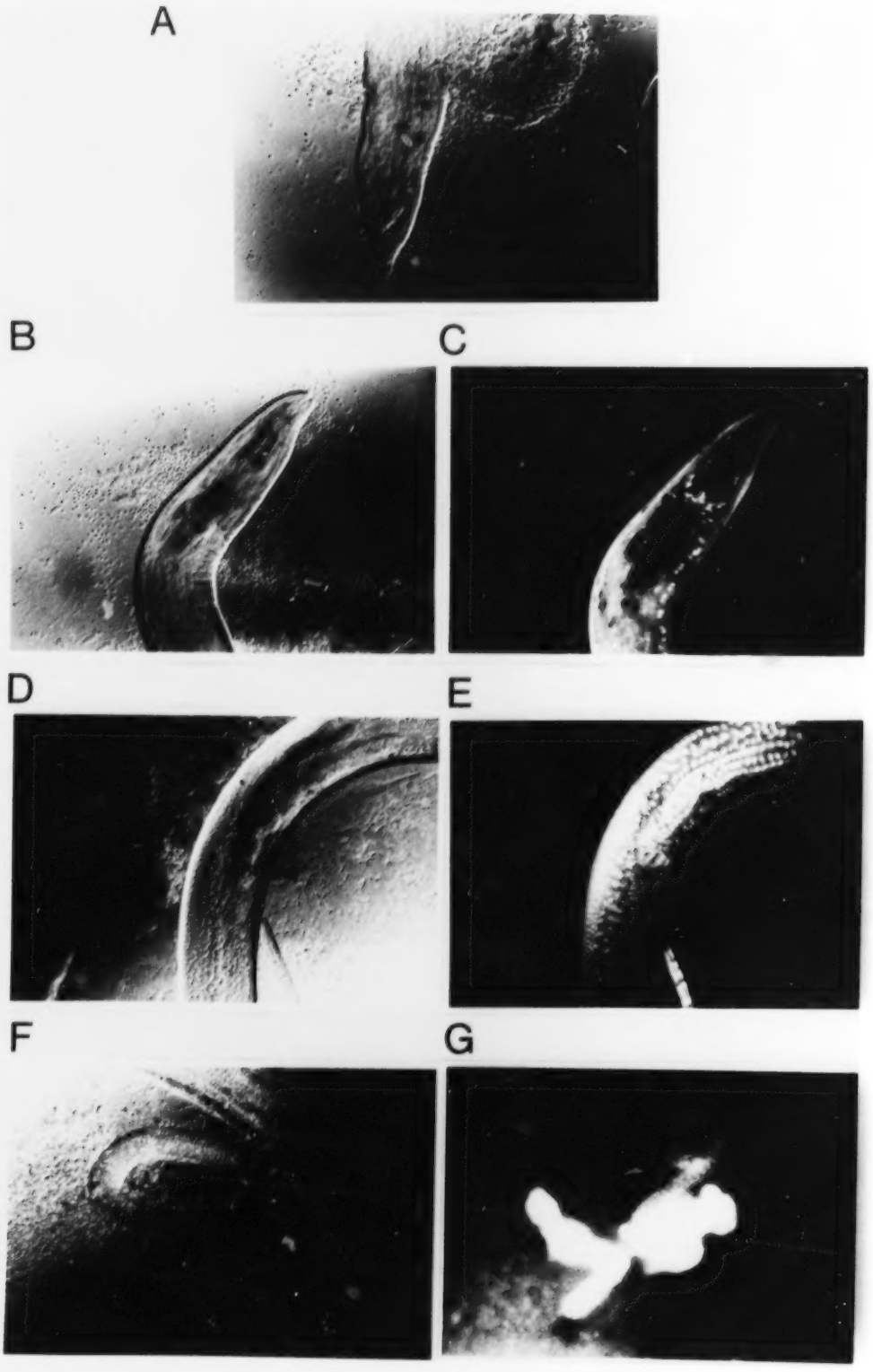

A
B
C
D
E
F
G

Figure 13: Composite photomicrographs using Nomarski optics of an XO transgenic adult (DP90, *edEx26*, pDP#TJ01) showing feminization of the germline. Features to note are the wild-type looking XO tail and the production of oocyte-like cells in the germline (see Figure 1 for a diagram of a wildtype XO animal) .

Figure 14: Photomicrographs of a transgenic XO animal (DP90 *edEx26*, pDP#TJ01). Higher magnification photomicrographs of the gonad region showing oocyte production (A), wildtype XO tail development (B) and the endo-reduplicated cells in the gonad (C, stained with DAPI).

A

B

C

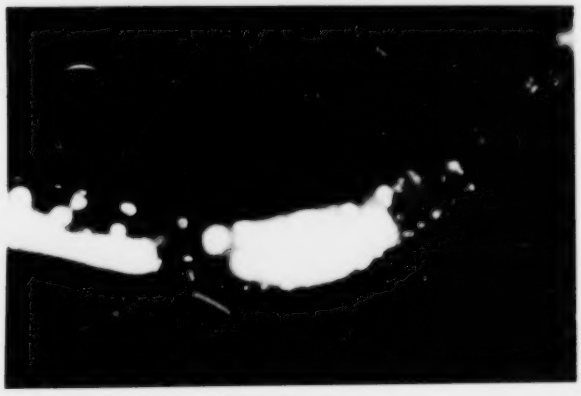


Figure 15: βGal staining patterns observed in *fem-1(hc17ts)* mutants carrying *edEx26* at 20°C. Panels A and B show βGal staining in the distal tip cells of XO animals similar to that seen in DP98 XO animals. Panels C and D show staining in the distal tip cells of XX animals. B and C are also visualized using DAPI staining in order to locate the position of the nuclei.

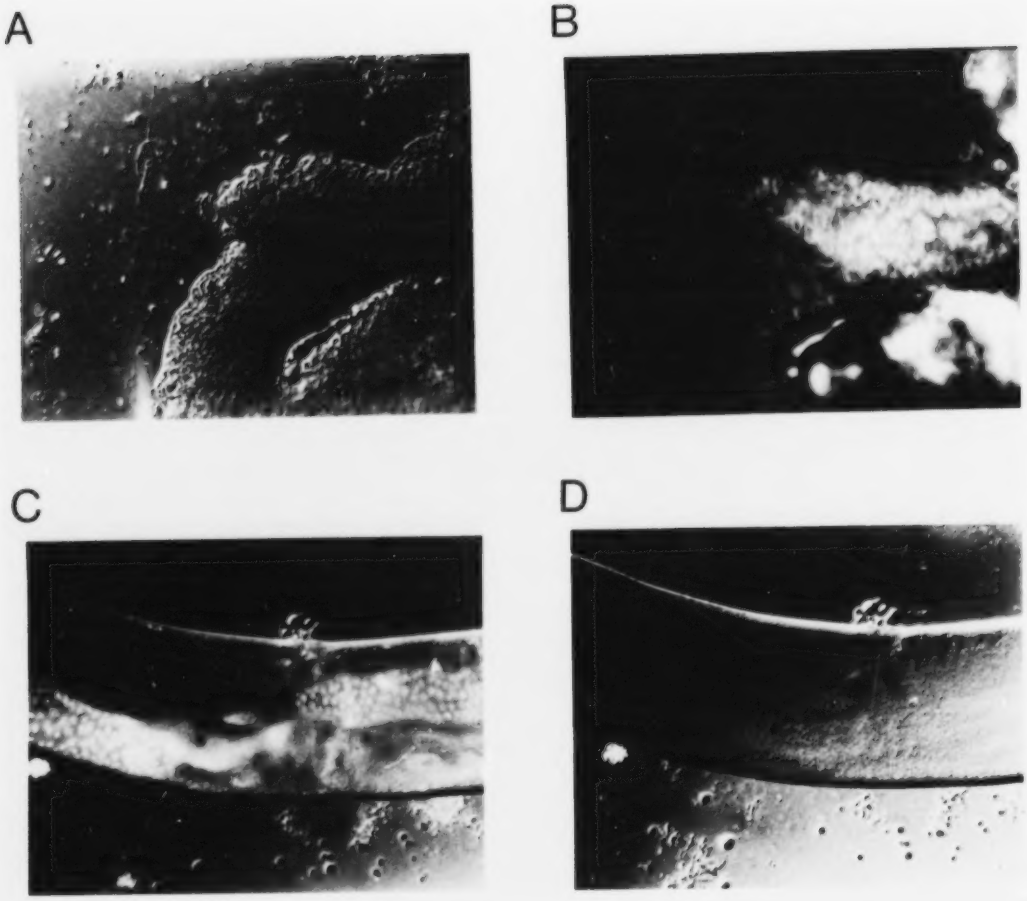
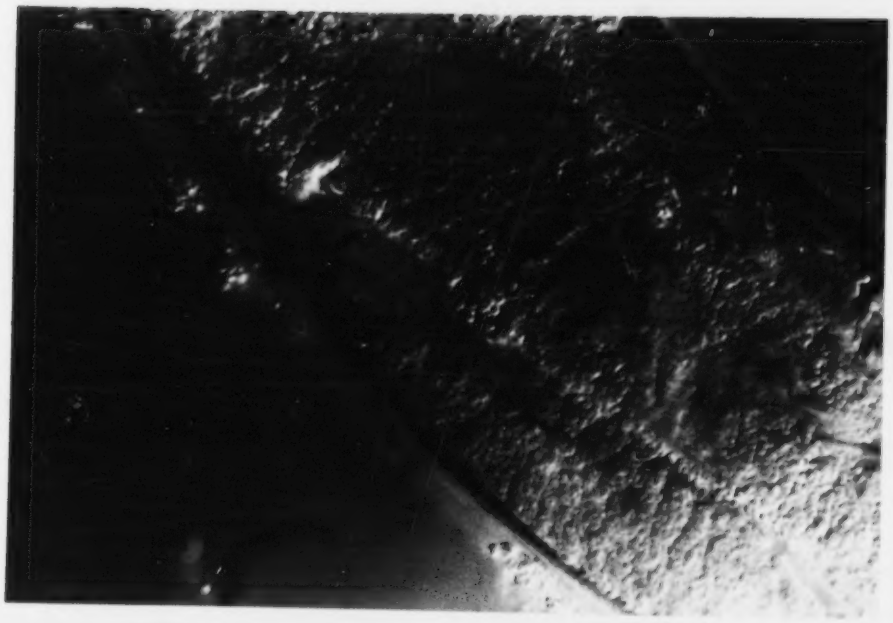

A
B
C
D

Figure 16: βGal staining patterns observed in *fem-3(q20gf,ts)* XX mutants carrying *edEx26* at 25°C. A and B, higher magnifications of the distal tip cell (indicated by the arrow) at the end of one gonad arm in an XX animal showing masculinization of the germline. B is also visualized using DAPI staining in order to locate the position of the nuclei.

A



B

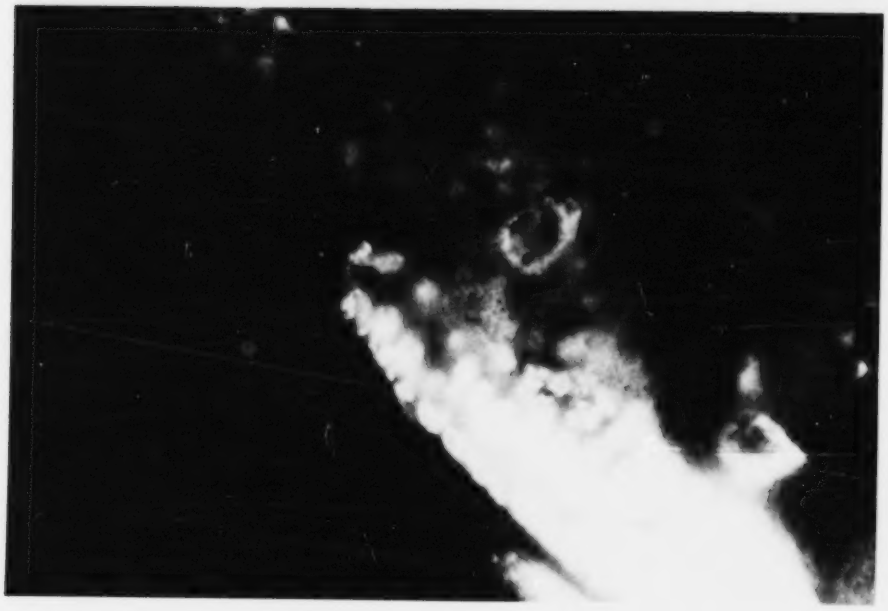
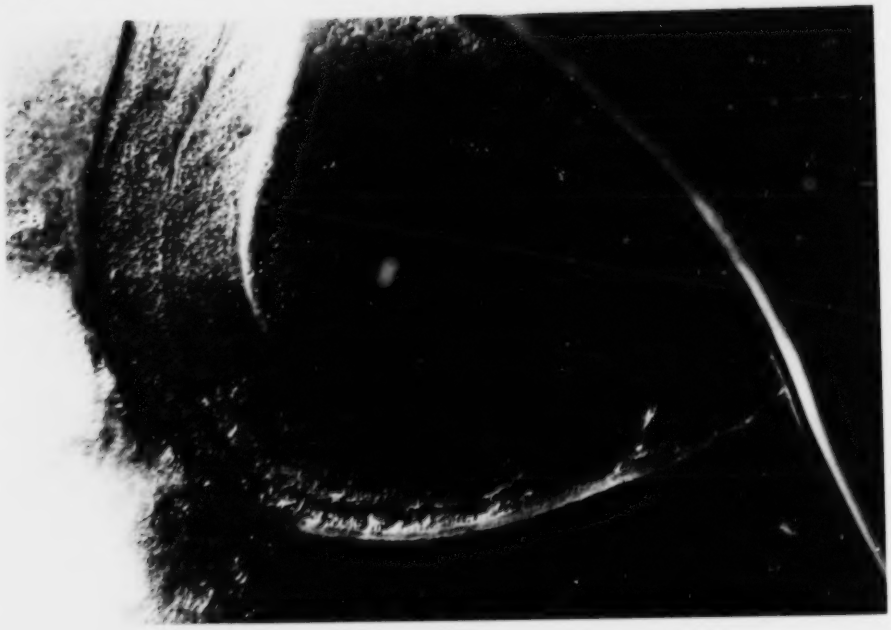


Figure 17: βGal staining pattern observed in a *tra-2(b202ts)* XX mutant carrying *edEx26* raised at 25°C. A and B show no staining in the distal tip cells of the gonad. B also demonstrates that these animals are producing sperm in their germline (see arrow) which are represented the small DAPI staining cells.

A



B

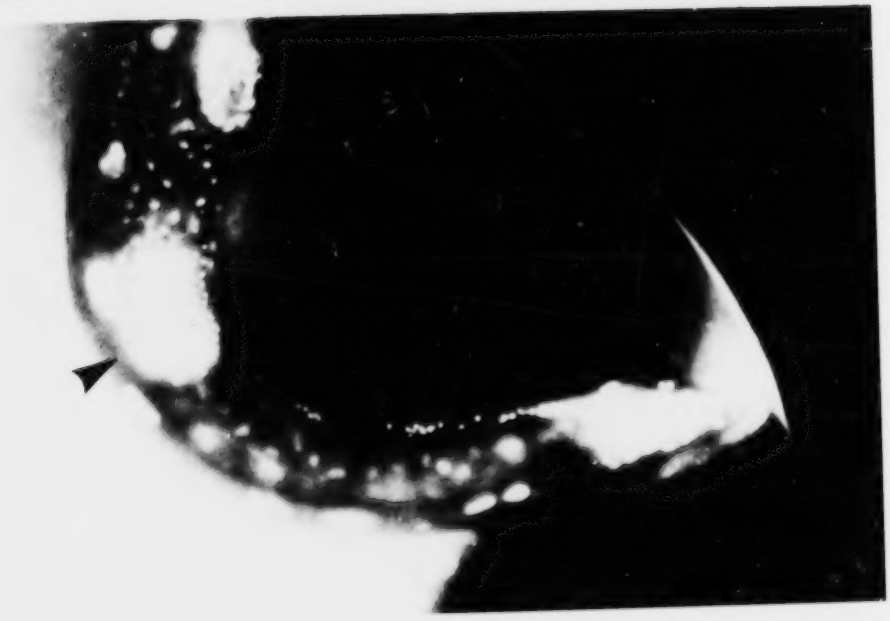


Figure 18: Diagram showing a possible model for how FEM-2 controls the spermatogenesis/oogenesis choice from the distal tip cells of the male. *glp-1* and *lag-2* are involved in the decision to switch from mitosis to meiosis and the *fem* genes are involved in whether those cells develop as sperm or oocytes. This diagram shows the first meiotic cells developing as sperm due to packaged product (F) and later cells due to a secreted 'sperm determining factor'. The arrows from the distal tip cells (DTC) indicate the secretion of some factor that promotes spermatogenesis.

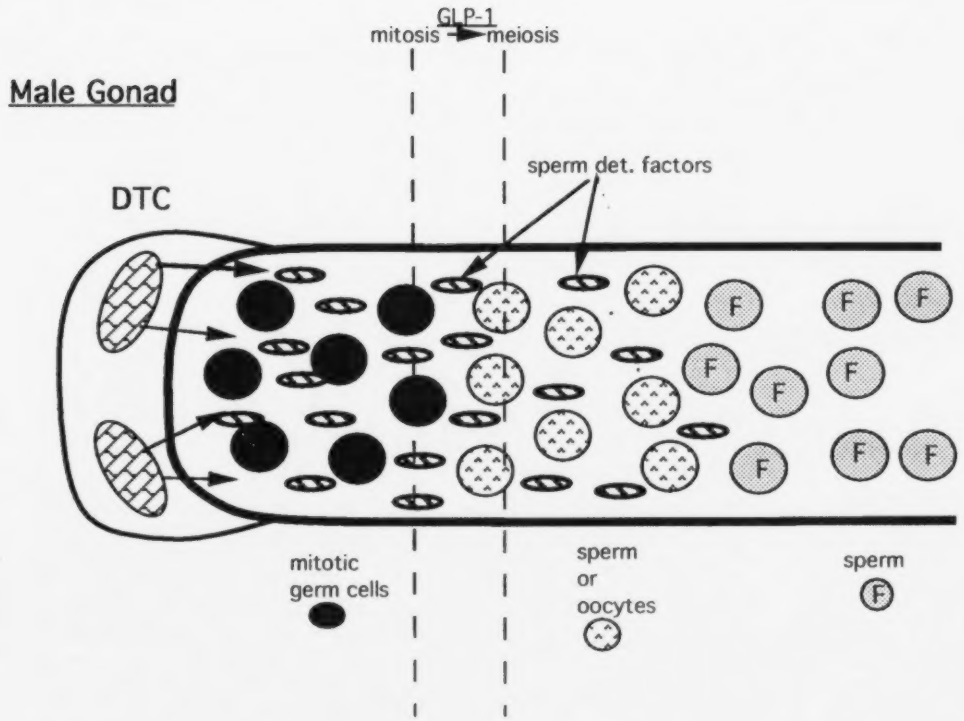
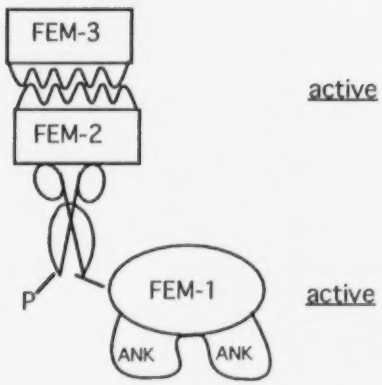
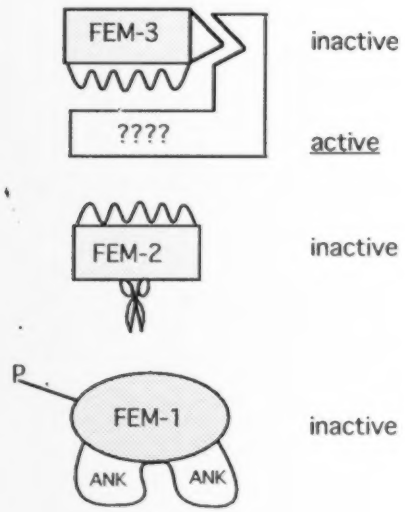

GLP-1
mitosis
meiosis
Male Gonad
sperm det. factors
DTC
F
mitotic
germ cells
sperm
or
oocytes
sperm
F

Figure 19: Diagram showing a possible mode of interaction of the FEM proteins in the development of the germline. During male development FEM-3 binds to FEM-2 and activates it which then allows FEM-2 to dephosphorylate FEM-1. During female development, one of the MOG proteins prevents FEM-3 from binding FEM-2, which prevents the activation of FEM-1. Stippled boxes indicate inactive proteins, ANK represents the protein binding motifs of FEM-1. This diagram does not show FEM-1's germline target as it is unknown.

Male Development



Female Development



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Appendix

Worm Lysis Buffer
50 mM KCl
10 mM Tris (pH 8.2)
2.5 mM $MgCl_2$
0.45% NP-40
0.45% Tween 20
0.01% gelatin
0.06% mg/ml proteinase K

10X PCR Reaction Buffer
100 mM Tris (pH 8.3)
500 mM KCl
15 mM $MgCl_2$
0.01% gelatin

5X Transcription Buffer
200 mM Tris (pH 7.5)
30 mM $MgCl_2$
10 mM spermidine
50 mM NaCl

Guest Buffer
4M Guanidine isothiocyanate
50mM Tris (pH 7.4)
10 mM EDTA
0.5% N-Lauroylsarcosine
1% v/v 2-Mercaptoethanol

RNA Probe-Northern Hybridization Solution
50% formamide
5XSSPE
5X Denhardts
5% SDS
0.1 mg/ml salmon sperm DNA

DNA Probe-Northern Hybridization Solution
50% formamide
5X SSPE
5X Denhardts
0.1 % SDS
0.2 mg/ml salmon sperm DNA

RNA Loading Buffer
360 ul deionized formamide
80 ul 10X Mops Buffer
130 ul formaldehyde (37%)9-
50 ul H_2O
50 ul ethidium bromide (10 mg/ml)
40 ul glycerol
40 ul saturated Bromophenol Blue

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750 ul

X-gal Staining Solution
33 mM NaH2PO4
166 mM Na2HPO4
1 mM MgCl2
5mM K4Fe(CN)6
40 ug/ml SDS
240 ug/ml Xgal
0.6% N,N-dimethylformamide
75 ug/ml kanamycin sulphate
2 ug/ml DAPI